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Host-parasite interaction during tularemia in the mouse

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HOST-PARASITE INTERACTION DURING TULAREMIA IN THE MOUSE

by

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Host-Parasite Interaction During Tularemia in the Mouse

Director: J. Frederick Bell

The subject of host-parasite interaction during tularemia is controversial. Uncertainites exist regarding the nature of the host response to tularemic infection, and regarding the means by which the etiologic agent causes disease. The purpose of this study was to investigate the basis of these uncertainties and to attempt resolution of them.

Although Francisella tularensis has generally been considered to be a facultative intracellular parasite, the results obtained in this investigation indicate that this organism is in extracellular association with host mononuclear phagocytes during the disease process in the mouse; and in this respect, may be unique among facultative intracellular organisms. The following lines of evidence indicate an extracellular nature of this organism with regard to mouse peritoneal macrophages: 1.) F. tularensis strain Schu S4 was indifferent to the increased bactericidal capacity of a BCG activated RES in the mouse, 2.) when injected into the peritoneal cavity of mice, F. tularensis became associated with the fluid protion of the peritoneal exudate, whereas, Listeria monocytogenes, when injected ip., became associated with the cellular portion of the exudate, and 3.) fluorescent antibody staining of macrophage cultures inoculated with F. tularensis strain Schu S4, did not reveal the presence of intracellular antigen.

Specific antibodies have been believed to have an insignificant role in the expression of immunity to fully virulent tularemia organisms. The results presented in this study do not support this belief. The following lines of evidence indicate that antibodies may be important in resistance: 1.) specific humoral immunity in combination with a nonspecifically activated RES resulted in a degree of protection against challenge with F. tularensis strain Schu S4, 2.) ^{51}Cr-release methodology showed that immune serum influenced the cytopathic effect of F. tularensis strain Schu S4 on cultured macrophages, 3.) pretreatment of the organism with immune serum prior to ip. injection, increased bacterial association with peritoneal cells, and 4.) immune serum enabled cultured macrophages to suppress the growth of the organism in cell cultures.

In the mouse, F. tularensis strain Schu S4 may not ordinarily be ingested by phagocytes, and may be able to overcome host defenses by extraphagocytic proliferation and dissemination to sites of specific target tissues. Resistance to infection appears to require the synergistic cooperation of both humoral and cell-mediated immune mechanisms. Specific antibodies control dissemination by promoting phagocytosis, while specifically-sensitized macrophages destroy ingested organisms by virtue of some specific altered, intrinsic quality.

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ABBREVIATIONS

BCG	*Mycobacterium bovis* strain bacille Calmette-Guerin
C	degrees Centigrade
CFU	colony forming units
DH	delayed hypersensitivity
EEA	ether-extracted antigen
GCBA	glucose-cysteine blood agar
g	gravities
im.	intramuscularly
ip.	intraperitoneally
iv.	intravenously
Klett xxx	suspension adjusted to a density of xxx Klett units on a Klett-Summerson photoelectric colorimeter fitted with a #42 blue filter
LD_{50}	50% lethal dose
LVS	*Francisella tularensis*, live vaccine strain
mg	milligram
ml	milliliter
mm	millimeter
MTD	mean time of death
μci	microcurie
μg	microgram
μm	micrometer
PBS	phosphate buffered saline
PMN	polymorphonuclear neutrophil

PPD	purified protein derivative (from tubercle bacilli)
RES	reticuloendothelial system
RPMI	Roswell Park Memorial Institute
sal-gel	saline-gelatin solution
S.E.	standard error
sc.	subcutaneously
T-cell	thymus derived lymphocyte
UV	ultraviolet

CHAPTER I

INTRODUCTION

Francisella tularensis is a minute, Gram-negative, pleomorphic, nonmotile, aerobic bacterium (16). It was first isolated in 1912 by McCoy and Chapin while investigating a plague-like disease among ground squirrels in Tulare County, California (81). In 1921, Francis named this disease "tularemia" (45).

Tularemia is a zoonosis. It is primarily a disease of wild rodents and other small animals. It is also widespread among arthropod parasites. Burroughs et al. (18) have compiled a list of 48 naturally infected vertebrates. Man is only incidentally involved and is usually a dead-end host. Man-to-man transmission is rare. The disease can present itself in a variety of clinical forms. In animals, tularemia is often an acute, febrile and fatal disease, however, subclinical infections and even chronic carrier states are not uncommon. In man, tularemia may be a protracted and debilitating disease characterized by fever, nausea, headache, malaise, chills and enlargement of the lymph nodes, or it may be a subacute, inapparent disease identified only in retrospect by serological procedures (96).

It is of the utmost importance to specify the virulence of the particular strain of F. tularensis whenever reference to the organism is made. Numerous strains of F. tularensis exist which differ in their relative virulences (9). Strains of high virulence are such that as few as 1 to 10 organisms may be sufficient to result in a fatal infection of susceptible animals. Strains of low virulence,

even when administered in large qunatities, may be inocuous and used as vaccines. An accepted procedure for quantitation of the virulence of F. tularensis was set forth by Bell et al. (9). Because various strains can be expected to react with hosts differently, specification of virulence is, therefore, critical when investigating host interaction with F. tularensis.

Coupled with the high virulence of F. tularensis is the high infectivity of this organism. R. R. Parker, as cited by Foshay (44), related that he knew of no other disease of animals transmissible to man by means so numerous and diverse. Tularemia has ranked high on the list of laboratory-acquired infections (98).

Infection may readily be initiated by contact with the organism. F. tularensis has been reported to be able to even penetrate the unbroken skin (46,103). Infected animals constitute a major source of infection and serve to transmit the disease to those who may ingest or come into contact with such sources, e.g. hunters, trappers, meat processors, housewives (96,138).

Arthropods are vectors of infection for man and may also aid in perpetuating the reservoir. Fleas, flies, ticks and mosquitos have been implicated as responsible for many reported cases of tularemia in man (7,19,48,54). It is of epidemiological interest that the greatest incidence of tularemia in the United States in the fall and winter months in noted in the Eastern region of the country, while the greatest incidence of the disease in the Western region is noted in the spring and summer months. These seasonal occurrences are attributed to the popularity of rabbit hunting in the East and to

the tick season in the West.

Another means by which the desease can be transmitted is by drinking contaminated water (19,48,103). Bell and Stewart have with regularity, isolated F. tularensis from streams in the Bitterroot Valley of Western Montana. The organism has been shown to remain viable in natural waters for extended periods, especially in the winter (10). Infective water is another means by which the reservoir is perpetuated.

Tularemia may also be contracted via the respiratory route (82). Inhalation of dust from hay or straw contaminated with animal feces often results in a pulmonary form of the disease (27). An aerosol created by the sneezing of an infected laboratory animal has purportedly resulted in one known case of tularemia (Bell, personal communication).

F. tularensis has been considered to be a member of the group of organisms collectively referred to as facultative intracellular parasites. Such organisms are readily ingested by the phagocytic cells of the reticuloendothelial system (RES). They are able to resist intraphagocytic destruction in nonimmune hosts. Pathogenicity is related to their ability to survive and proliferate within host cells (122). Other bacteria in this group include species of Brucella, Listeria, Pasteurella, Mycobacterium, Salmonella and Yersinia.

Resistance to intracellular infection is dependent on the functional capacity of the host cells to inactivate the ingested organism (77,80,125); a manifestation of acquired cellular immunity

(86,87). This type of immunity involves a complex interaction between lymphoid cells in the host, and is most often achieved by immunization with viable attenuated organisms (22,76). Mackaness, a pioneer in advancing the concept of cell-mediated immunity, discovered that in mice, immunity to L. monocytogenes could be passively transferred to naive recipients with viable spleen cells from immune donors (79). Numerous other studies have also shown that immunity to intracellular organisms can be transferred with viable lymphoid cells (1,47,63,78,84,127,136). The importance of cellular factors in immunity to facultative intracellular parasites is also indicated by an association between resistance and the state of delayed hypersensitivity (DH) (21,23,28,63,77,123,124). Acquired cellular immunity and DH are thought to have similar or closely related mechanisms (28,77,97). Resistance to facultative intracellular parasites, however, has also been demonstrated in the absence of DH (2,36,37,53,77,106,137). The immunosuppressive effect of anti-thymocyte globulin on immunity to numerous intracellular organisms has implicated the thymus-dependent lymphocytes (T-cells) as being the cell subpopulation mainly responsible for the state of acquired cellular immunity (20,21,50,68,80,84). A cell-mediated mechanism is believed to be responsible for the state of acquired cellular immunity to Brucella sp. (36,56,95), Listeria sp. (4,79), S. enteriditis (92,109) and S. typhimurium (12,12,76).

Cell-mediated immune responses usually require the participation of macrophages (52). Macrophages may act as accessory cells and function in the afferent arc of the immune response by processing

antigens. They may also function as an effector cell against invading organisms. Macrophages are often the effector cell in the immune response to facultative intracellular infections (108). A role for the mononuclear phagocyte was first postulated by Metchnikoff in the late nineteenth century. Since that time, much evidence has accunulated which documents the importance of the macrophage as the effector cell in the prevention of disease. Passive transfer of macrophages from resistant animals has been shown to confer increased resistance to disease on normal recipients (1,118), while the administration of antimacrophage agents to animals has been shown to increase host susceptibility to infection (58,70,95,129). Numerous in vivo and in vitro investigation have demonstrated that the phagocytes of immune animals have greater ingestive and digestive abilities than cells from nonimmune donors (89,125,126). Lurie (72,73) made the initial observation that macrophages from rabbits immune to tuberculosis ingested acid-fast bacilli at a greater rate than macrophages from nonimmune animals; and that they could inhibit bacterial growth when placed in the anterior chamber of the eye of a normal rabbit. Further work by Suter (121) showed that macrophages from guinea pigs infected with Mycobacterium bovis strain BCG were able to suppress the growth of tubercle bacilli in vitro. Mackaness (74) demonstrated that prior infection of mice with L. monocytogenes enabled their peritoneal macrophages to resist intracellular growth of the organism. The time of appearance of the resistant macrophages coincided with the disappearance of viable Listeria from the liver and spleen. Thorpe and Marcus (125) reported that rabbit peritoneal

macrophages, harvested from immunized animals, had greater cytopeptic activities than did the counterpart cells from normal rabbits. Ruskin et al. (108) demonstrated that peritoneal macrophages harvested from nonspecifically immunized mice were protected against necrotization by bacterial and protozoal challenge.

In its role as an effector cell, the macrophage derives its specificity from molecules liberated by specifically sensitized T-cells after antigenic stimulation of those cells. Interaction between T-cells and macrophages in cellular immune responses is mediated by these soluble substances which, in some unknown way, alter the physiology of other cells. Researchers have identified and characterized a wide range of these lymphoid products (97). The elaboration of these soluble mediators by antigen-stimulated lymphocytes is believed to be resposible for resistance to infection with facultative intracellular parasites.

Host infection with facultative intracellular organisms often results in the induction of nonspecific resistance. Appropriate stimulation of host cells may result in cross-protection against infection with numerous organisms that have a predilection for intracellular existence (75,77,108). Nonspecific stimulation can be enacted by many types of intracellular infections (12,14,36,57,75) and also by nonviable agents such as mycobacterial antigens (2,5,32), lipopolysaccharides (15,100,107,115,135), simple lipids (25), surfactants (26) or complement components (131). Immunization of mice with viable BCG has been shown to result in resistance against infection with Brucella (120), Listeria (14) and Salmonella (111). This

phenomenon of heterologous resistance has been shown to extend beyond phylogenetic lines to include not only bacteria, but a number of protozoa (108), viruses (3,69) and neoplasms (67). This wide range of resistance is attributed to the activity of mononuclear phagocytes which have increased microbicidal capacities that are not directed exclusively against the original infecting organism (51,108). Macrophages possessing such activity are commonly referred to as being activated. Such cells in vitro are morphologically distinct from normal cells (14,75). They appear larger than normal and have increased invagination of their cell membrane. They contain greater than normal numbers of cytoplasmic digestive vacuoles and lysosomes. They have elevated levels of oxidative and hydrolytic enzymes. They attach more readily to glass and spread more extensively. The ability of these cells to react nonspecifically indicates that a common mechanism of immunity underlies intracellular infection. This mechanism has not been clearly defined.

Although there is little dispute that the effector cell responsible for immunity to facultative intracellular organisms is the mononuclear phagocyte, the role of specific antibodies in resistance is of considerable controversy among investigators. Some maintain that immunity is primarily due to a change in the intrinsic properties of the host cell and that antibodies are of only secondary importance in host resistance (12,20,56,75,76,89). Passive transfer of convalescent serum has been shown, in many cases, to be ineffective in protecting recipients against infection. Organisms in an intracellular environment are often shielded from humoral factors. The fact that nonbact-

erial substances are able to induce cross-protection against facultative intracellular parasitic infection has been used as supportive evidence for the contention that specific antibodies have no significant role in resistance to infection by this type of organism.

Other investigators, however, argue that antibodies have an important function in resistance to facultative intracellular parasites (36,37,104,105). Numerous *in vitro* studies have demonstrated that normal and immune macrophages are capable of dealing with such parasites only if immune serum is present in the culture medium. Phagocytosis and inhibition of growth is often less effective if specific antibodies are not present. Fong *et al.* (40,41) found that macrophages from BCG inoculated mice were resistant to the cytopathic effect of virulent tubercle bacilli only when serum from immune animals was present in the culture medium. Jenkin and Benaceraff (59) showed that pretreatment of virulent *S. typhimurium* with immune serum increased the rate at which the organisms were ingested and destroyed by macrophages *in vitro*. Jenkin and Rowley (60) reported that resistance to *S. typhimurium* can be passively transferred with immune serum if sufficient quantities were used. Immune serum has been shown to be required to prevent virulent *B. melitensis* from destroying normal and immune phagocytes *in vitro* (36,37).

The subject of host-parasite interaction during tularemia is controversial. Uncertainties exist regarding the nature of the host immune response to tularemic infection and regarding the means by which the etiologic agent causes disease.

Some of the factors involved in immunity against tularemia appear

similar to those which are important in resistance to other facultative intracellular organisms. Immunity can be passively transferred to naive recipients with viable lymphoid cells from immune donors, but not always with serum from such donors (1,108,118, 127,136). Viable vaccines are generally more immunogenic than killed vaccines (20,21,31,33,34,42,43,62,110,132). The onset of immunity to tularemia can be shown to coincide with the development of DH (20,21, 95) and the effector cell responsible for acquired immunity to this organism is believed to the the macrophage (21,89,95).

Immunity to tularemia is, however, unlike immunity to other facultative intracellular parasites in several interesting respects. Resistance to tularemic infection is specific. Claflin (20) and Claflin and Larson (21) have shown that the induction of nonspecific resistance in mice by immunization with viable BCG does not afford protection against challenge with fully virulent tularemia organisms. They have also shown that immunization with viable attenuated F. tularensis induces no protection against subsequent challenge with F. novicida or S. typhimurium. It is unusual that this facultative intracellular parasite is not only unaffected by nonspecific factors of immunity, but that it is also unable to induce such nonspecific immunity. Further investigation of these findings would be substantiated.

The protective efficacy of viable vs nonviable tularemia vaccines is an involved subject. To date, the only means of assuring maximum immunity has been immunization with living organisms (20,24). Russian researchers were the first to report that living avirulent organisms were more protective than killed preparations (128). Since that time,

the superiority of live vaccines has been well documented (20,21,31,33, 34,42,43,62,95,110,132). Protection from nonviable vaccines depends on the natural susceptibility of the animal receiving the vaccine and on the virulence of the challenge organism. Killed vaccines have been shown to be immunogenic in white rats (17,30,65) and also in mice and guinea pigs if challenged with strains of less than full virulence (8,42,66,90,93,113). In general, nonviable antigenic preparations are protective only 1.) in animals which have a degree of natural resistance, and 2.) when strains of low or moderate virulence are used as the challenge organism. Explanations of the underlying mechanism responsible for this variance in immunogenicity are scanty.

Disagreement exists regarding the role of specific antibodies in immunity to tularemia. It has generally been held that antibodies are only of secondary importance in the host response to tularemic infection. More recently, however, reports have appeared in the literature which suggest that antibodies may assume an active and important role in resistance. These reports have also challenged the contention that F. tularensis is in intracellular association with host phagocytes during the the disease process. Further investigation of this matter would be in order.

The F. tularensis - mouse system provides a particularily lucrative model for investigating host interactions with facultative intracellular organisms. There is little, if any, chance that laboratory animals have been previously exposed to thes agent: an important consideration when experimenting with Gram negative bacteria.

Small numbers of organisms are sufficient to result in infection and usually lead to an acute and fatal disease. Endotoxin is not a complicating factor in the pathogenesis of the disease. Except for a single report (64), no endotoxin-like material has been isolated or boivin antigen detected (100,101). Also, bacterial strains of varying degrees of virulence are available to the researcher, as are experimental hosts differing in susceptibility.

Statement of Problem

Francisella tularensis has generally been considered to be a facultative intracellular parasite whose pathogenicity may be a consequence of intraphagocytic multiplication. Specific antibodies have been considered to be only of secondary importance in host resistance. These beliefs have recently been challenged by reports which suggest that F. tularensis may overcome host defenses primarily by extraphagocytic growth and that antibodies may in fact, be essential for host resistance. The subject of host-parasite interaction during tularemia remains an area of controversy. This study was undertaken to investigate the basis from which ambiguities have arisen and to attempt resolution of them.

CHAPTER II

MATERIALS AND METHODS

Animals

Outbred Swiss-Webster mice between 4 and 8 weeks of age, obtained from the colony at Rocky Mountain Laboratory, U.S. Public Health Service, Hamilton, Montana, were used throughout this investigation.

Adult, New Zealand white rabbits, obtained from the above institution served as the source of sera for cell culture media and passive transfer studies. All animals were allowed food and water *ad libitum*.

Organisms

Strains of *F. tularensis* were obtained in lyophilized form from Dr. J. F. Bell, U.S. Public Health Service, Rocky Mountain Laboratory, Hamilton, Montana. The organisms were maintained on glucose cysteine heart agar (Difco) supplemented with fresh defibrinated rabbit blood to a final concentration of 5%. *F. tularensis* strain Schu S4, isolated by Foshay in 1941 is a strain of high virulence. It has a LD_{50} of 1-10 colony forming units (CFU) in mice, guinea pigs and rabbits. *F. tularensis* strain 425 F_4G was isolated by Bell (9) from a tick in Western Montana. It is a highly virulent strain in mice and guinea pigs (LD_{50}: 1-10 CFU), but of lower virulence in the rabbit. The live vaccine strain (LVS), developed by Russian scientists (128) and currently used in the United States as a live vaccine against

tularemia, is of low virulence in mice (LD_{50}: 10^4-10^5 CFU). Lyophilized lots of the various strains were established. Periodically, ampules were rehydrated and the contents cultured on glucose cysteine blood agar (GCBA) plates (29,49). Prior to use, the organisms were reisolated twice from experimentally-infected mice to ensure full virulence of the strain.

Suspensions of challenging or immunizing doses were prepared in sterile physiological saline-gelatin solution (0.1% gelatin & 0.15% NaCl) (sal-gel) from 24-48 hr cultures of the organism. The suspensions were adjusted to an appropriate concentration based on turbidometric readings in the Klett-Summerson photoelectric colorimeter fitted with a #42 blue filter. Concentrations of viable organisms in suspension were determined by standard plate counting techniques. One-tenth ml volumes of appropriate dilutions were plated on each of 3 GCBA plates and incubated at 37 °C for 24-48 hr. Klett-250 suspensions (zero dilution) of F. tularensis routinely contained 10^9-10^{10} CFU per ml.

A culture of Mycobacterium bovis strain BCG-Paris was obtained through the courtesy of Dr. C. L. Larson, Stella Duncan Memorial Research Institute, University of Montana, Missoula, Montana. The organism was maintained as described below.

Cultures of L. monocytogenes and S. typhimurium were kindly provided by Mr. Richard L. Hodinka, Department of Microbiology, University of Montana, Missoula, Montana and maintained on Trypticase-Soy agar (Difco) plates at 4 C. The organisms has mouse LD_{50} values of $10^{3.7}$ and $10^{2.0}$ CFU respectively.

A culture of F. novicida was obtained from the stock culture collection at Rocky Mountain Laboratory, U.S. Public Health Service, Hamilton, Montana, and maintained on GCBA plates. This organism had a mouse LD_{50} of $10^{2.7}$ CFU.

Vaccines

Ether-extracted antigen (EEA) was prepared from F. tularensis strain Schu S4 according to the method of Larson (65). Flasks containing Snyders peptone broth (116) were inoculated with the organism and incubated at 37 °C for 48 hr on a shaker. Cells were harvested by centrifugation at 8,000 x g for 30 min and washed 3 times in sal-gel. The sedimented cells were then resuspended in sal-gel and treated with an equal volume of ethyl ether for 24 hr at room temperature with occasional shaking. The ether phase was drawn off and gaseous nitrogen passed through the aqueous phase to evaporate residual ether. The remaining solution, now designated EEA, has been reported to be a crude suspension of polydisperse cell wall fragments (90). The suspension was then adjusted to a Klett reading of 100 with sal-gel and stored a 4 °C in sealed serum vials. Absence of viable organisms was confirmed by plate culture and by mouse inoculation. An immunizing dose consisted of 0.2 ml, and was injected into a tail vein of the mice. Antigenicity was confirmed by a positive capillary tube precipitation reaction and by titer determinations on injected animals.

Viable BCG was prepared according to a protocol maintained at the Stella Duncan Memorial Research Institute, University of Montana,

Missoula, Montana. Flasks of Dubos medium (Difco) were inoculated with Mycobacterium bovis strain BCG-Paris, from a culture maintained on Hohns medium (Difco). The cultures were incubated at 37 °C for 7-9 days, passed to fresh Dubos medium and allowed to incubate another week. The cells were then harvested by centrifugation at 4,080 x g for 30 min and washed 3 times in Dubos medium. The cells were resuspended in Dubos medium and adjusted to a density of 200 Klett units. The vaccine was stored at -70 °C in sealed serum vials. Purity was checked by Ziehl-Nielson acid-fast staining. The working vaccine was readied by diluting the suspension 1:1 with Dubos medium. A standard immunizing dose of 0.2 ml was injected into a tail vein of the mice. This amount corresponded to approximately 250 μg wet weight of bacteria and 7×10^7 CFU. Efficacy of this vaccine was tested by injecting groups of mice and 3 weeks post-injection, determining the presence of 1.) splenomegaly, 2.) delayed hypersensitivity to purified protein derivative (PPD), and 3.) nonspecific resistance.

Serologic tests

Bacterial agglutination titers were conducted on serum samples according to standard tube agglutination procedures. A stock suspension of formalin-killed F. tularensis strain 425 F_4G was prepared (approximately 1×10^9 cells per ml) and used as the agglutinogen. The stock suspension was diluted 1:300 to obtain the working solution. Serial 2-fold dilutions (0.5 ml) were made for each serum sample with saline, and equal volumes of the agglutinogen were added to each tube. The agglutination titer was determined as the

reciprocal of the highest dilution of serum showing positive agglutination after overnight incubation at 37 °C.

Passive transfer of sera

New Zealand white rabbits were injected sc. with approximately 1 x 10^6 viable F. tularensis strain Schu S4 organisms and streptomycin therapy was initiated 3 days later (daily im. injections of 200 mg streptomycin sulfate for 5 days). When recovery was assured, the rabbits were anesthesized with sodium pentobarbital and exsanguinated. Sera were separated and pooled. Volumes of 0.5 ml were injected into a tail vein of the recipient mice 1 hr before bacterial challenge and again at 1 hr after challenge.

Protection tests

Protection against a challenge infection was measured in terms of survival after injection with serial 10-fold dilutions of challenge organism into separate groups of mice. Experimental and control groups of 10-15 mice each were injected sc. with 0.5 ml of each dilution. Deaths among challenged mice were recorded daily for 14 days. The LD_{50} and the mean time to death (MTD) were calculated for each group.

In vitro studies

Mouse peritoneal adherent cells were used in in vitro correlate experiments to host-parasite interaction. To collect the cells, mice were killed by cervical dislocation and injected ip. with 5.0 ml of Dulbecco's phosphate buffered saline (PBS) containing 5 units of heparin per ml. Eliciting agents or irritants were not used to obtain

higher yields of cells because it has been demonstrated that results will vary with different elliciting agents (24,38,83). After gentle kneading of the abdomen, the surrounding skin was swabbed with 70% ethyl alcohol and reflected back. The peritoneal sac was punctured aseptically and the enclosed fluid was allowed to drain into sterile, cold, siliconized, glass centrifuge tubes. Bloody exudates were discarded. Following centrifugation at 500 x g for 5 min, the supernatant fluid was discarded. Sedimented cells were washed 3 times in Dulbecco's PBS + heparin, treated with Tris-NH_4Cl for 10 min at room temperature and washed again. The cells were then suspended to the desired seeding concentration in cell culture mainenance medium (RPMI supplemented with 20% heat inactivated rabbit serum, 200 units/ml penicillin and 2.5 μg/ml fungizone). Cell suspensions were placed in appropriate culture vessels and allowed to incubate for 45 min at 37 °C in a 5% CO_2 atmosphere. The cultures were then washed to remove nonadherent cells.

The *in vitro* experimental setup (Fig. 1) was designed to correlate with antecedent *in vivo* experiments. Two variables existed; 1.) the immune status of the macrophage source, and 2.) the type of serum present in the cell culture medium. The cell cultures were inoculated with *F. tularensis* strain Schu S4 and two parameters were investigated; 1.) the fate of the macrophages, and 2.) the fate of the inoculum.

In vitro cytopathology assays

Cell count method - Mouse peritoneal macrophages were cultured in

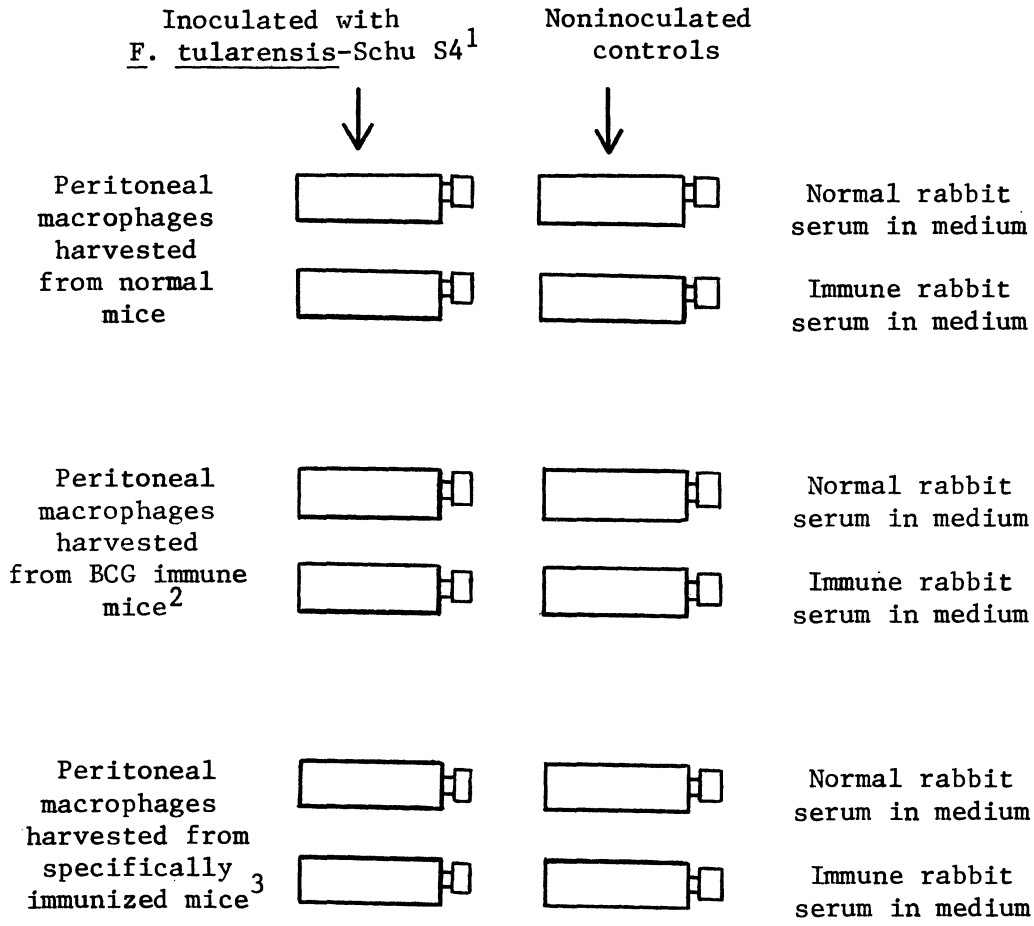

Fig. 1. Experimental setup used in determining the effect of macrophage source and specific antibody on macrophage viability and bacterial growth in cell cultures inoculated with F. tularensis strain Schu S4.

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[1]Cultures were inoculated at a ratio of approx. 10 bacteria/macrophage.

[2]Mice were immunized iv. with viable BCG (7 x 10^7 CFU, 250μg wet wt) 3 weeks prior to harvesting of macrophages.

[3]Mice were given a 1° injection sc. of approx. 10^3 viable LVS organisms and a 2° injection 2 weeks later of approx. 10^6 viable Schu S4 organisms. Macrophages were harvested 3 weeks after 2° injection. GCBA plate cultures of harvested cells at this time yielded no viable bacteria.

[4]Serum was obtained from rabbits which had been injected sc. with approx. 10^6 viable Schu S4 organisms and treated with streptomycin (daily im. injections of 200 mg streptomycin sulfate, initiated 3 days post-injection).

Lab-Tek® cell culture chamber slides. Prior to seeding of the cells, circles of approximately 1 mm in diameter were etched on the surface of the glass floor of each culture chamber by means of a diamond-point pencil and a template. The etching fragments were removed and the cell culture chamber slides were disinfected by UV irradiation. Macrophage cultures were established in the chambers. Photomicrographs were made of the cells contained within the inscribed area of the chamber with a Nikon® model F2 inverted microscope coupled to a polaroid photoattachment adapter cone fitted with a 1.3X lens and a 4" x 5" type 55 PN back and an AFM exposure control. Macrophage cultures were inoculated with F. tularensis strain Schu S4 at a ratio of approximately 10 bacteria per macrophage. Photomicrographs of the circumscribed cells were made daily for 7 days. Cultures were washed before photographing to remove cells which had become nonadherent. Cell counts were conducted on the photomicrographs, and the decrease in the number of viable, adherent cells was recorded.

^{51}Cr-release method - Mouse peritoneal exudate cells were harvested and processed as previously described. The cells were incubated in the presence of radioactive $Na_2{}^{51}CrO_4$ at a concentration of approximately 100 μci/10^6 macrophages in a 37 °C water bath for 30 min with occasional shaking. After 3 washings in cold RPMI medium to remove nonincorporated label, the cells were resuspended in RPMI medium and seeded at a concentration of approximately 10^6 cells per ml. in plastic screw-top test tubes. Following an incubation period of 45 min, the nonadherent cells were removed by washing. Radioisotopic counts were made after withdrawing the culture medium and inserting

the culture vessel into a Nuclear® model 8775 gamma counter. Culture medium was replaced after the readings. Macrophage cultures were inoculated with F. tularensis strain Schu S4 and counts of the radioactivity remaining in the adherent cells were made at 12 hr intervals for 5 days. Background radiation was counted and recorded. Cytopathology was measured by determining the rate at which the label was released from the adhered macrophages.

In vivo phagocytosis of bacteria by mouse peritoneal cells

In vivo phagocytosis of F. tularensis and L. monocytogenes by mouse peritoneal cells was determined by the method of Taplits and Myrvik (124). Bacterial suspensions were injected ip. into groups of 5 mice each. One hr after injection, the peritoneal cavities were lavaged and the exudates were centrifuged in siliconized centrifuge tubes at 480 x g for 5 min. Cell-associated and supernatant-associated bacteria were assayed by plating serial dilutions of the respective fractions. The data are presented as the percent of the total bacteria recovered.

Immunofluorescence

Fluorescein isothiocyanate-conjugated antitularensis immunoglobulin had been prepared by Mr. Scott J. Stewart, Rocky Mountain Laboratory, U.S. Public Health Service, Hamilton, Montana, according to the method of Peacock, et al. (94), and was used to stain macrophages from cultures which had been inoculated with F. tularensis. Macrophage cultures were inoculated with F. tularensis at a ratio of approximately 50 bacteria per macrophage. The cells were incubated

until cytopathology was first noted. The cultures were then washed, and the cells were fixed in acetone at -70 °C for at least 12 hr. The cells were stained as described in a protocol by Bell (unpublished) and viewed in a Leitz-Wetzlar Ortholux II/Diavert fluorescent microscope equipped with a 35 mm Orthomat W camera. Appropriate controls were included.

CHAPTER III

RESULTS

Attempt to induce nonspecific immunity to F. tularensis strain Schu S4

This study stemmed from the finding by Claflin and Larson (21) that immunization of white mice with viable BCG afforded no cross-protection against subsequent challenge with F. tularensis strain Schu S4. Viable BCG, when administered to animals, typically results in nonspecific stimulation of the recipients' RES. Such stimulation has been shown to be effective in inducing protection against infection with numerous etiologic agents. It appeared unusual that F. tularensis should be virtually unaffected by nonspecific factors of immunity.

A preliminary experiment was conducted to confirm this earlier finding of Claflin and Larson (21). Groups of mice were injected iv. with viable BCG (7×10^7 CFU, 250 μg wet wt). After a period of 3 weeks, when nonspecific resistance was at its height (D. Lodmell and C. L. Larson, unpublished data), groups of mice were challenged sc. with serial 10-fold dilutions of F. tularensis strain Schu S4. At this time, it was determined that the inoculated mice had a 4.7X splenomegaly compared to noninoculated controls. Attempts to demonstrate DH to PPD were unsuccessful. Separate groups of mice were also challenged with serial 10-fold dilutions of L. monocytogenes, S. typhimurium, and with F. novicida. Table I shows that inoculation of mice with viable BCG did not result in protection against challenge with F. tularensis strain Schu S4. Results shown in Table II indicate that this BCG vaccine was capable of inducing nonspecific resistance.

Table I. Attempt to induce nonspecific resistance to sc. challenge with F. tularensis strain Schu S4 in mice immunized 3 weeks previously with viable BCG (7.0×10^7 CFU, 250 μg wet wt) injected iv.

Number of challenge organisms (CFU)	BCG immunized mice		Control mice	
	d/t[a]	MTD[b]	d/t	MTD
10^1	10/10	5.7 ± 0.7	10/10	5.1 ± 0.6
10^2	10/10	5.0 ± 0.7	10/10	5.0 ± 0.0
10^3	10/10	4.5 ± 0.5	10/10	4.5 ± 0.5
10^4	10/10	4.3 ± 0.7	10/10	4.1 ± 0.6
10^5	10/10	4.1 ± 0.3	10/10	4.1 ± 0.3
10^6	10/10	4.2 ± 0.5	10/10	4.0 ± 0.0
10^7	10/10	3.8 ± 0.8	10/10	3.1 ± 0.7
10^8	10/10	3.5 ± 0.5	10/10	3.1 ± 0.6
LD_{50}	$< 10^1$		$< 10^1$	
$Logs_{10}$ protection	0			

[a]Deaths per total mice challenged.

[b]Mean time of death (days) ± S.E.

Table II. Ability of viable BCG to induce nonspecific resistance to sc. challenge with L. monocytogenes, S. typhimurium and F. novicida[a].

Number of challenge organisms (CFU)	Challenge organism					
	L. monocytogenes		S. typhimurium		F. novicida	
	Control	BCG Immune	Control	BCG Immune	Control	BCG Immune
10^1	0/5[b]	0/5	1/5	0/5	0/5	0/5
10^2	1/5	0/5	3/5	0/5	3/5	0/5
10^3	3/5	0/5	4/5	1/5	3/5	0/5
10^4	2/5	1/5	5/5	3/5	4/5	0/5
10^5	4/5	0/5	5/5	3/5	5/5	0/5
10^6	4/5	0/5	4/5	3/5	5/5	1/5
10^7	5/5	1/5	5/5	5/5	4/5	3/5
10^8	5/5	2/5	5/5	5/5	5/5	4/5
LD_{50}	$10^{3.7}$	$>10^{8.0}$	$10^{2.0}$	$10^{4.4}$	$10^{2.7}$	$10^{6.8}$
$Logs_{10}$ protection	> 4.3		2.4		4.1	

[a]Mice were challenged sc. 3 weeks after iv. injection of viable BCG (7.0×10^7 CFU, 250 μg wet wt).

[b]Deaths per total mice challenged.

Effect of BCG immunization on infection with F. tularensis strains of reduced virulence

Highly virulent F. tularensis strain Schu S4 has been shown to be unaffected by nonspecific factors of immunity. It was desired to know whether this finding applies to F. tularensis strains of reduced virulence. The preceeding experiment was repeated using F. tularensis strain 425 F_4G and strain LVS as the challenge organisms. Data presented in Table III show that nonspecific activation of the RES resulted in a degree of protection against the strains of reduced virulence. Although BCG immunization did not protect against mortality in mice challenged with F. tularensis 425 F_4G, a significant increase in the mean time of death was noted. Protection against challenge with F. tularensis strain LVS was manifest as both a decrease in mortality and an increase in the mean time of death of the fatalities. Coupled with the results presented in Table I, it can be observed that the degree of protection against tularemic infection, attributable to RES activation, is inversely related to the virulence of the particular infecting strain. Subsequent investigation will be centered primarily on host interaction with F. tularensis strain Schu S4.

Table III. Protective effect of immunization with viable BCG on mice challenged sc. with F. tularensis strain LVS and strain 425 F_4G[a].

Number of challenge organisms	Strain of F. tularensis							
	LVS				425 F_4G			
	Control		BCG immune		Control		BCG immune	
	d/t[b]	MTD[c]	d/t	MTD	d/t	MTD	d/t	MTD
10^1	0/10	-	0/10	-	10/10	6.4 ± 0.5	10/10	8.8 ± 0.8
10^2	0/10	-	0/10	-	10/10	6.2 ± 0.4	10/10	8.1 ± 1.2
10^3	3/10	7.6 ± 0.6	0/10	-	10/10	5.8 ± 0.4	10/10	7.8 ± 1.2
10^4	8/10	6.3 ± 0.9	0/10	-	10/10	5.4 ± 0.7	10/10	7.2 ± 1.8
10^5	10/10	6.4 ± 1.0	0/10	-	10/10	5.1 ± 0.3	10/10	6.9 ± 1.3
10^6	10/10	5.0 ± 0.8	4/10	6.8 ± 0.5	10/10	4.5 ± 0.7	10/10	5.8 ± 1.2
10^7	10/10	4.4 ± 0.8	9/10	5.1 ± 0.6	10/10	4.2 ± 0.4	10/10	6.6 ± 1.6
10^8	10/10	4.2 ± 0.8	10/10	4.7 ± 0.5	10/10	3.7 ± 0.3	10/10	5.6 ± 1.4
LD_{50}	$10^{3.4}$		$10^{6.2}$		$< 10^{1.0}$		$< 10^{1.0}$	
$Logs_{10}$ protection	2.8				0			

[a]Mice were challenged sc. 3 weeks after iv. injection of viable BCG (7×10^7 CFU, 250 μg wet wt).

[b]Deaths per total mice challenged.

[c]Mean time of death (days) ± S.E.

The combined effect of specific humoral immunity and nonspecific RES activation on resistance of mice to F. tularensis strain Schu S4

C. L. Larson (personal communication) suggested that a BCG activated RES might require the presence of specific antibodies for the expression of resistance to infection with fully virulent strains of F. tularensis. In the following 2 experiments, the combined effect of specific antibodies and BCG immunization on the resistance of mice to F. tularensis strain Schu S4 was determined. A humoral response was established by: 1.) active immunization with a bacterial antigen, or 2.) passive administration of immune serum.

Experiment 1. - Specific active immunization with EEA combined with nonspecific RES activation by viable BCG.

Groups of mice were injected with EEA, BCG or both. Mice were inoculated with 0.2 ml of a Klett-100 suspension of EEA, followed by a similar injection 2 weeks later. Agglutination tests with a F. tularensis antigen revealed that the response of the mice to EEA was highly variable (Table IV). Mice inoculated with EEA were challenged sc. with serial 10-fold dilutions of F. tularensis strain Schu S4 1 week after the second inoculation. To investigate the combined effect of a specific and nonspecific vaccine, mice, which had been inoculated with a primary dose of EEA 2 weeks previously, were injected iv. with a Klett-200 suspension of viable BCG that had been diluted 1:1 with a Klett-100 suspension of EEA. The mice were challenged 3 weeks later as described above.

The results from a typical experiment are shown in Table V and Fig. 2. None of the immunization regimes resulted in protection against

Table IV. Antibody response of mice to primary and secondary immunization with EEA[a].

Sample number	Agglutination titer	
	2 weeks post-1° injection	1 week post-2° injection
1	<4	16
2	8	128
3	8	4
4	<4	32
5	4	32
6	4	64
7	<4	128
8	8	128
9	4	64
10	4	32

[a]Mice were injected iv. with 0.2 ml of a Klett-100 suspension of EEA and reinjected 2 weeks later with an equal dose.

Table V. Effect of specific immunization with EEA and nonspecific immunization with viable BCG on mice challenged sc. with serial 10-fold dilutions of F. tularensis strain Schu S4.

Number of challenge organisms	Treatment							
	Control		EEA		BCG		EEA & BCG	
	d/t[a]	MTD[b]	d/t	MTD	d/t	MTD	d/t	MTD
10^1	10/10	5.1 ± 0.6	10/10	6.3 ± 1.2	10/10	5.7 ± 0.7	10/10	10.2 ± 1.8
10^2	10/10	5.0 ± 0.0	10/10	5.4 ± 0.8	10/10	5.0 ± 0.7	10/10	7.9 ± 1.1
10^3	10/10	4.5 ± 0.5	10/10	5.0 ± 0.7	10/10	4.5 ± 0.5	10/10	7.3 ± 1.4
10^4	10/10	4.1 ± 0.6	10/10	5.1 ± 0.9	10/10	4.3 ± 0.7	10/10	6.9 ± 0.9
10^5	10/10	4.1 ± 0.3	10/10	4.9 ± 1.6	10/10	4.1 ± 0.3	10/10	6.1 ± 0.3
10^6	10/10	4.0 ± 0.0	10/10	4.8 ± 0.8	10/10	4.2 ± 0.5	10/10	6.4 ± 0.7
10^7	10/10	3.1 ± 0.7	10/10	3.8 ± 0.9	10/10	3.8 ± 0.8	10/10	6.2 ± 0.8
10^8	10/10	3.1 ± 0.6	10/10	3.7 ± 0.8	10/10	3.5 ± 0.5	10/10	5.1 ± 0.6
LD_{50}	$< 10^1$		$< 10^1$		$< 10^1$		$< 10^1$	

[a]Deaths per total mice challenged.

[b]Mean time of death (days) ± S.E.

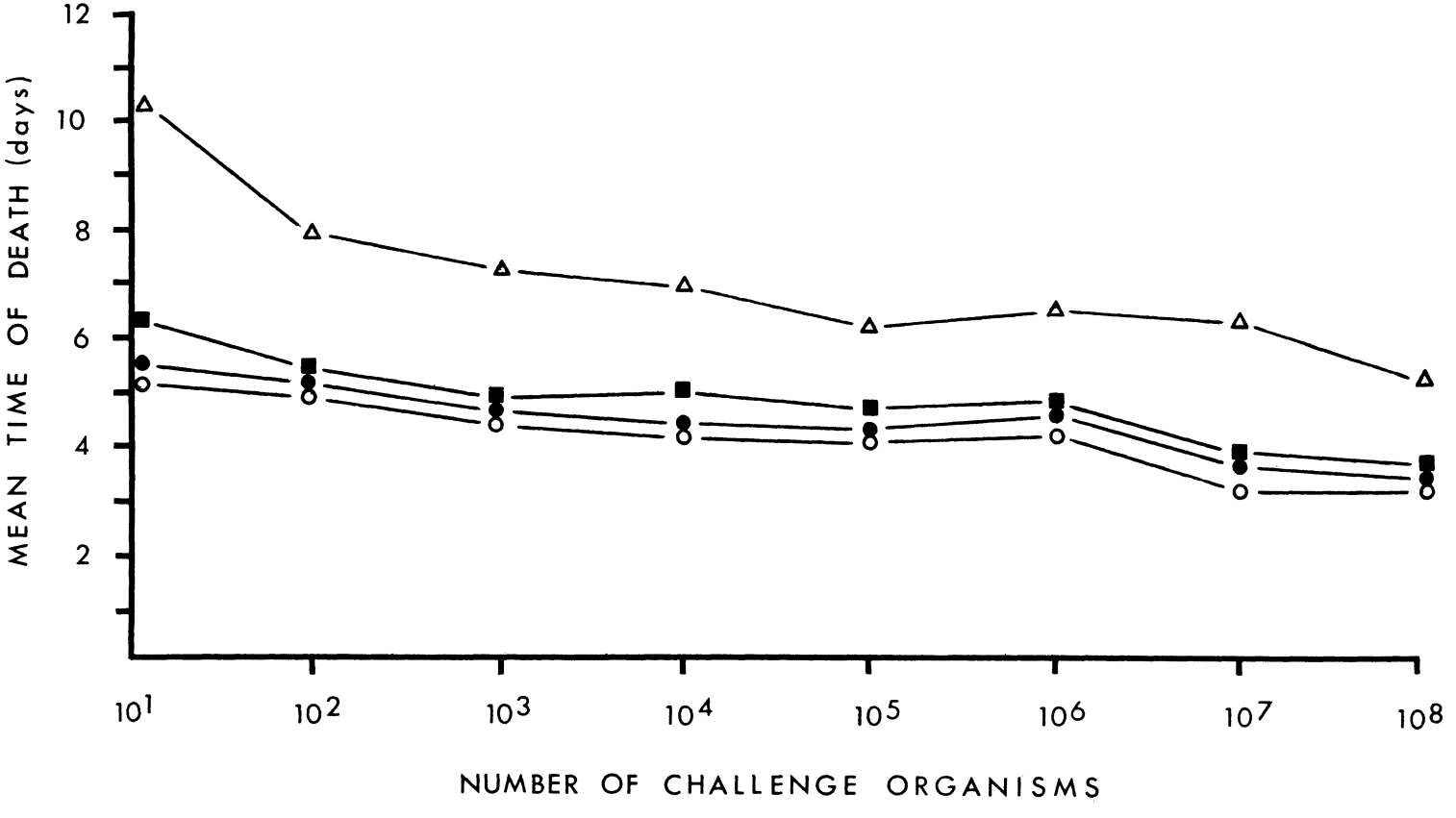


Fig. 2. Effect of specific immunization with EEA and nonspecific immunization with viable BCG on the mean time of death of mice challenged sc. with serial 10-fold dilutions of F. tularensis strain Schu S4. Normal mice o——o ; EEA immune mice ■——■ ; BCG immune mice ●——● ; EEA and BCG immune mice Δ——Δ .

mortality. Specific active immunization in combination with nonspecific immunization with viable BCG, however, resulted in a low order of protection as expressed by a statistically significant increase in the mean time of death of the challenged mice.

Experiment 2. - Passive transfer of immune serum combined with nonspecific RES activation by viable BCG.

As in Experiment 1, four experimental groups existed: 1.) an immunologically naive, control group of mice; 2.) a group of mice with specific circulating antibodies; 3.) a group of mice which had a BCG activated RES; and 4.) a group of mice with both specific humoral antibodies and a nonspecifically activated RES. Immune rabbit serum (agglutination titer: 1024) was injected in 0.5 ml amounts into a tail vein of recipient mice 1 hr before challenge with serial 10-fold dilutions of F. tularensis strain Schu S4, and again at 1 hr after challenge. Normal rabbit serum was likewise injected as a control into appropriate recipient groups. To investigate the combined effects of specific antibodies and a nonspecifically stimulated RES, sera were passively transferred to recipient mice which had been inoculated 3 weeks previously with viable BCG. The rate of clearance of rabbit antibodies from recipient mice was determined (Table VI and Fig. 3).

The results presented in Table VII and Fig. 4 essentially parallel the results of the previous experiment. None of the immunization regimes protected against mortality, however, degrees of protection were detected by observing the mean time of death of

Table VI. Antitularensis titer determinations on mice, following passive transfer of immune rabbit serum[a].

Sampling time[b]	Agglutination titer
0 hr	512
3 hr	512
6 hr	512
12 hr	256
1 day	256
2 day	256
3 day	128
4 day	128
5 day	128
6 day	128
7 day	128
8 day	64

[a]Mice were injected iv. with two 0.5 ml volumes of rabbit antitularensis serum, separated by a 2 hr interval (agglutination titer; 1024).

[b]Time 0 hr: immediately following second serum injection.

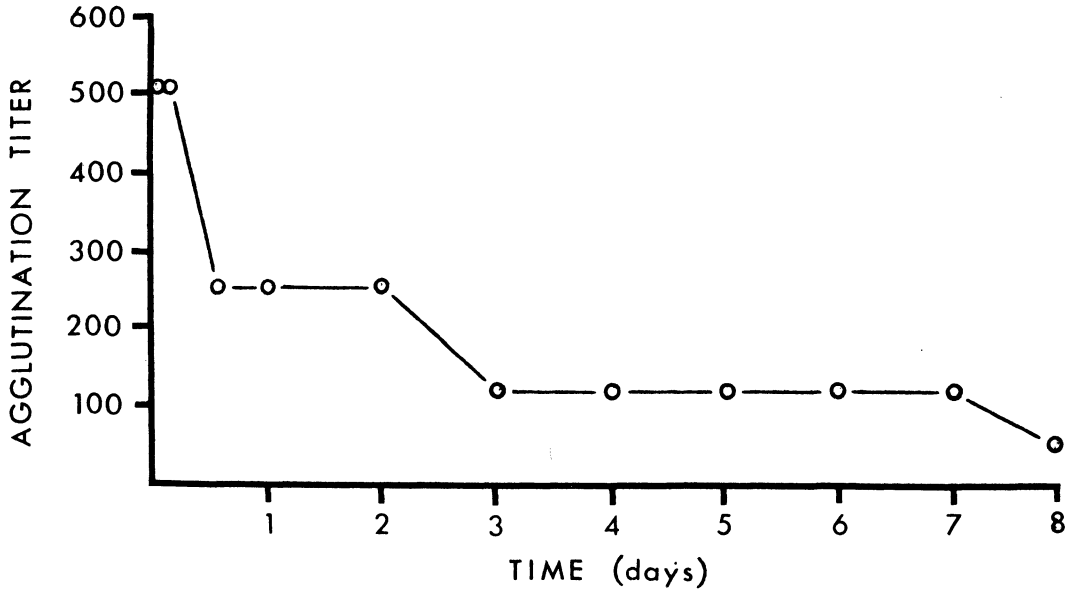


Fig. 3. Clearance of rabbit antitularensis immunoglobulin from recipient mice. Mice were injected iv. with two 0.5 ml volumes of rabbit antitularensis serum (separated by a 2 hr interval). Time 0: immediately following second serum injection. Titers were determined on sera pooled from 5 mice per sampling time.

of the fatalities. There was a slight degree of protection noted among the group of normal mice receiving immune serum attributable to the effect of the specific antibodies. RES activation alone resulted in no significant deviation from control values. Specific antibodies in conjunction with nonspecific RES activation afforded the greatest degree of protection to the challenged mice as revealed by prolongation of survival.

Table VII. Effect of passive transfer of immune rabbit serum and nonspecific immunization with viable BCG on mice challenged sc. with serial 10-fold dilutions of F. tularensis strain Schu S4.

Number of challenge organisms	Normal mice				BCG immune mice			
	Normal serum		Immune serum		Normal serum		Immune serum	
	d/t[a]	MTD[b]	d/t	MTD	d/t	MTD	d/t	MTD
10^1	10/10	5.4 ± 0.5	10/10	8.2 ± 0.8	10/10	6.6 ± 0.7	10/10	11.8 ± 2.4
10^2	10/10	5.0 ± 0.7	10/10	7.6 ± 0.6	10/10	5.6 ± 0.6	10/10	10.8 ± 1.9
10^3	10/10	4.6 ± 0.9	10/10	7.2 ± 0.5	10/10	5.8 ± 0.5	10/10	8.6 ± 1.8
10^4	10/10	4.2 ± 0.5	10/10	6.6 ± 1.1	10/10	4.6 ± 0.6	10/10	8.0 ± 1.2
10^5	10/10	4.2 ± 0.5	10/10	5.4 ± 0.6	10/10	5.0 ± 0.0	10/10	7.8 ± 1.1
10^6	10/10	4.0 ± 0.0	10/10	5.8 ± 0.5	10/10	4.0 ± 0.7	10/10	7.0 ± 1.4
10^7	10/10	3.2 ± 0.5	10/10	4.8 ± 0.8	10/10	4.2 ± 0.5	10/10	6.4 ± 1.1
10^8	10/10	3.0 ± 0.0	10/10	5.0 ± 0.0	10/10	3.6 ± 0.6	10/10	6.7 ± 1.0
LD_{50}	$<10^1$		$<10^1$		$<10^1$		$<10^1$	

[a]Deaths per total mice challenged.

[b]Mean time of death (days) ± S.E.

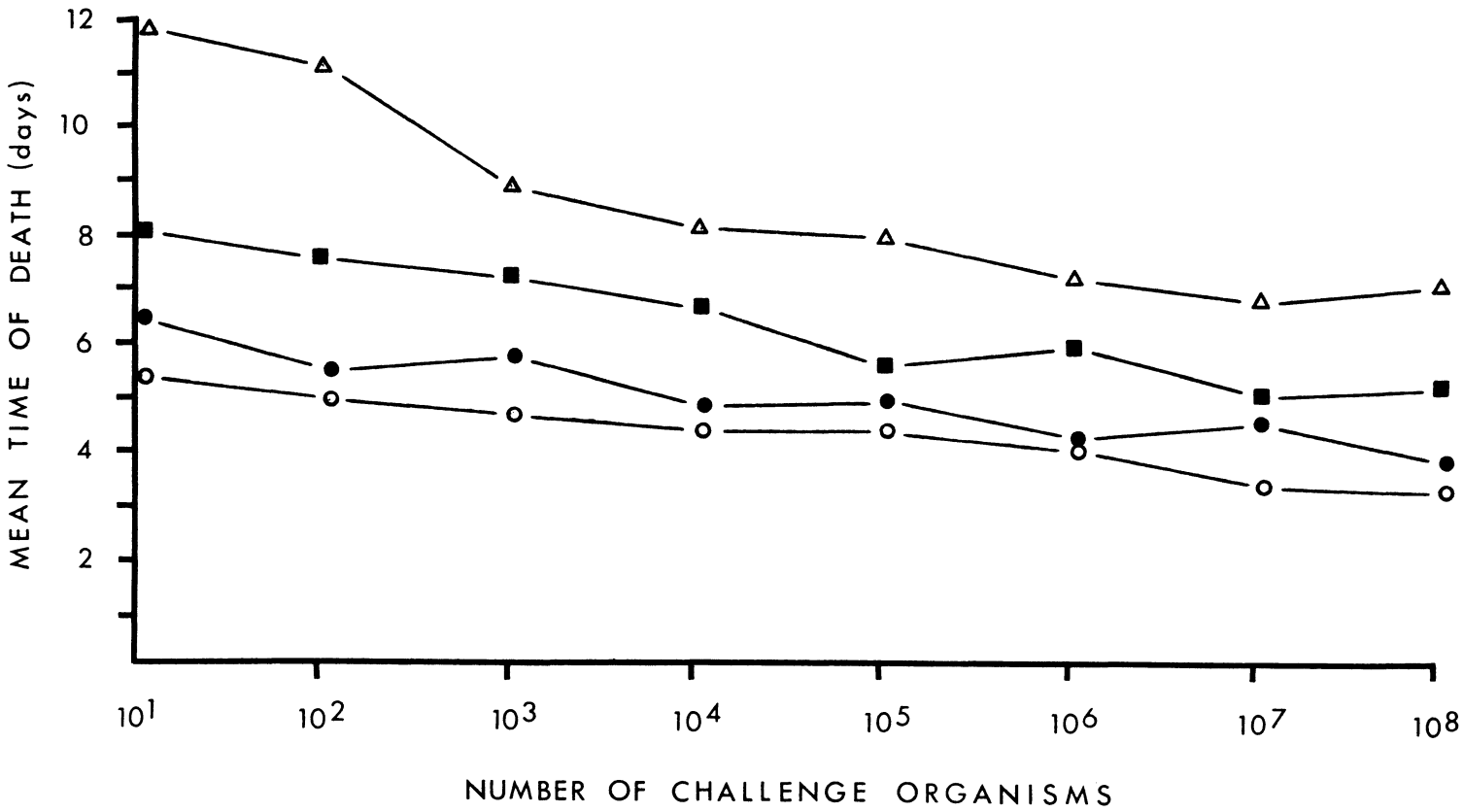


Fig. 4. Effect of passive transfer of immune rabbit serum and nonspecific immunization with viable BCG on the mean time of death of mice challenged sc. with serial 10-fold dilutions of F. tularensis strain Schu S4. Normal mice, normal serum o——o; normal mice, immune serum ■——■; BCG immune mice, normal serum ●——●; BCG immune mice, immune serum △——△.

In vitro correlates to host-parasite interaction

The preceeding *in vivo* studies produced unusual and interesting results. Further investigation was conducted *in vitro* in order to obtain more experimental control over this host-parasite system. Macrophages were used as the cell type in this *in vitro* system because it is generally believed that the prime mediator of protection against facultative intracellular bacterial infections is a cellular immune mechanism residing in the macrophage population.

Differential cell counts, conducted on Wrights and Giemsa stained cell cultures, revealed that the cell population, as determined by morphological criteria, consisted of greater than 95% mononuclear leucocytes (Fig. 5). Functional testing by latex particle ingestion showed that greater than 95% of the cells were phagocytic (Fig. 6). It is with these qualifications that the cells are referred to as macrophages.

Experiment 1. - Determination of macrophage viability via cell counts after inoculation of cultures with *F. tularensis* strain Schu S4.

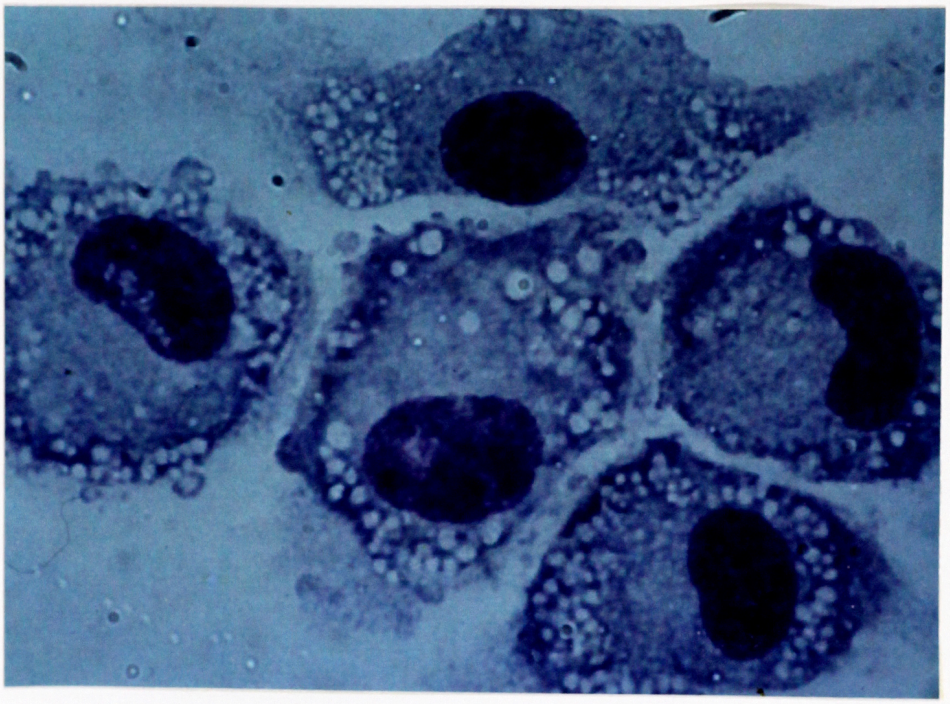
Cell counts of adherent (viable) macrophages were conducted daily on photomicrographs of cells which were contained within etched areas on the culture vessel floors. A typical photomicrograph, from which counts were made, is presented in Fig. 7. Cell counts were made with the aid of an acetate sheet overlay and a marking pen. The number of cells adherent within the enscribed area was monitored for 5 days.

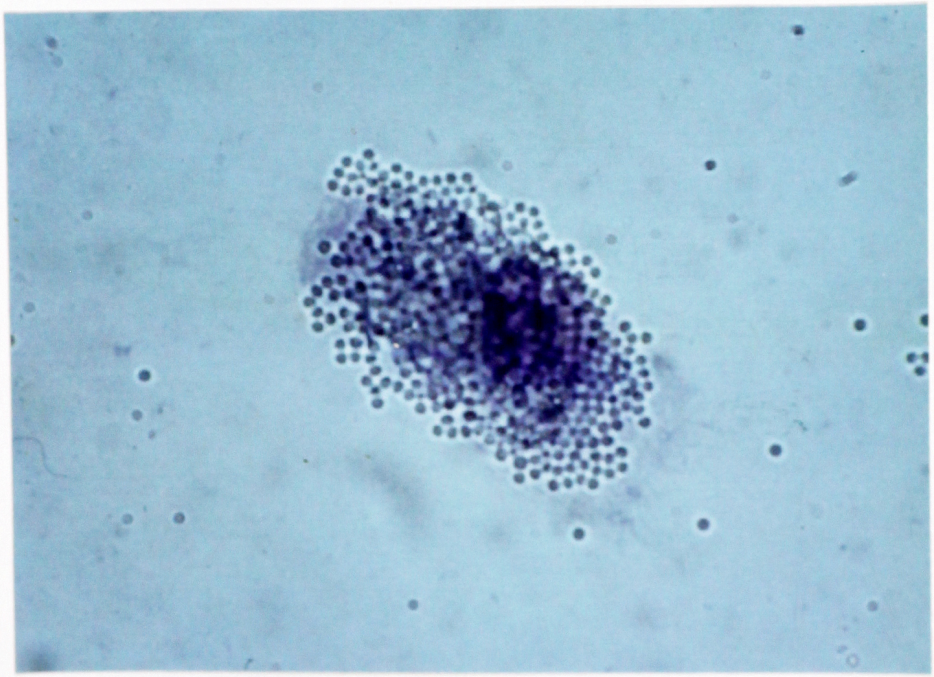
Results are shown in Table VIII and Fig. 8-10. This particular assay method apparently lacked sensitivity as evidenced by deviations

in cell counts and by wide fluctuations in the slope of the corresponding graphs. The assay was not repeated an adequate number of times to determine the standard error because of reason presented below. Conclusions from the results of this experiment were not made. The results do, however, evoke inferences that F. tularensis strain Schu S4 had a cytopathic effect on macrophages harvested from normal and BCG immunized mice, but not on macrophages harvested from mice specifically immunized with viable tularemia organisms.

Fig. 5. Mouse peritoneal adherent cells maintained in RPMI medium containing 20% rabbit serum, and stained by a modified Giemsa method. 2000X.

Fig. 6. Mouse peritoneal adherent cells maintained in RPMI medium containing 20% rabbit serum, 5 min after inoculation with 1.0 μm diam. polystyrene latex particles, and stained by a modified Giemsa method. 2000X.





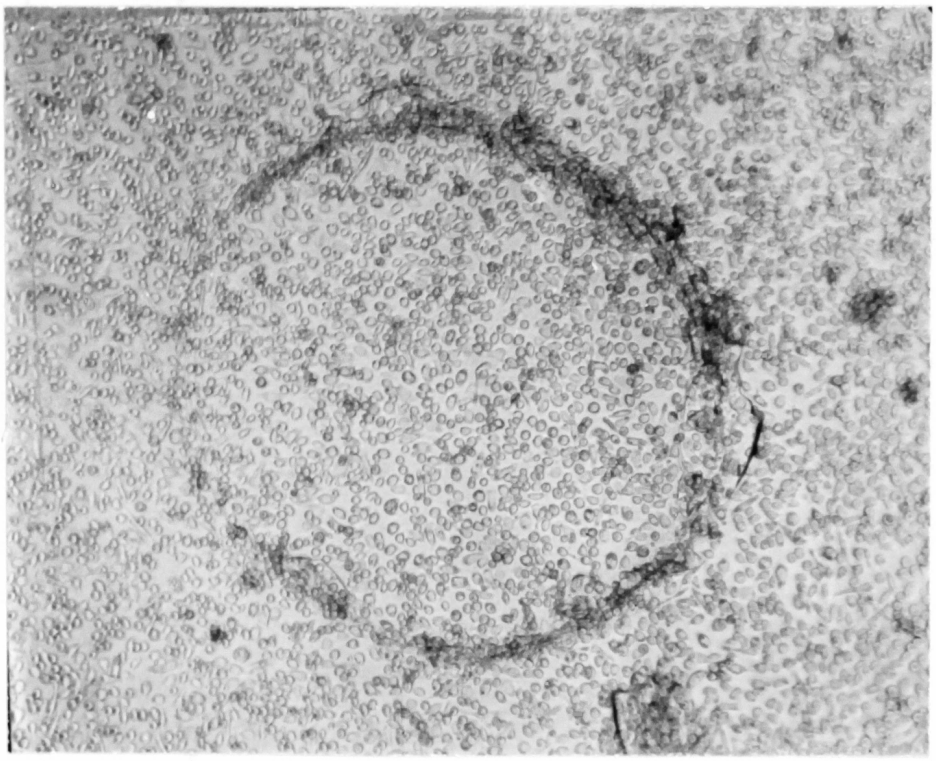


Fig. 7. Mouse peritoneal adherent cells maintained in RPMI medium containing 20% rabbit serum, as they appeared at time of challenge, with delineated area etched in the floor of the culture vessel. 50X.

Table VIII. Effect of F. tularensis strain Schu S4 on the viability of macrophages of various immune status, cultured in medium containing normal vs immune rabbit serum.

Macrophage source	Type of rabbit serum	Experimental group	Days post-inoculation 0	1	2	3	4	5
Nonimmunized mice	Normal	Control	1,507[a]	1,403	1,426	1,310	1,273	1,256
		Inoculated	1,694	1,385	882	729	702	676
	Immune	Control	1,618	1,515	1,355	1,429	1,504	1,467
		Inoculated	1,550	1,264	1,056	584	699	572
BCG immunized mice	Normal	Control	1,708	1,363	1,208	1,472	1,361	1,377
		Inoculated	1,420	1,021	826	688	838	641
	Immune	Control	1,575	1,654	1,351	1,440	1,299	1,223
		Inoculated	1,625	1,694	972	615	392	327
Specifically immunized mice	Normal	Control	1,599	1,422	1,457	1,329	1,198	1,133
		Inoculated	1,437	1,344	1,407	1,250	1,037	969
	Immune	Control	1,537	1,207	1,443	1,381	1,337	1,235
		Inoculated	1,667	1,595	1,526	1,603	1,550	1,474

[a]Number of macrophages adhered within delineated area.

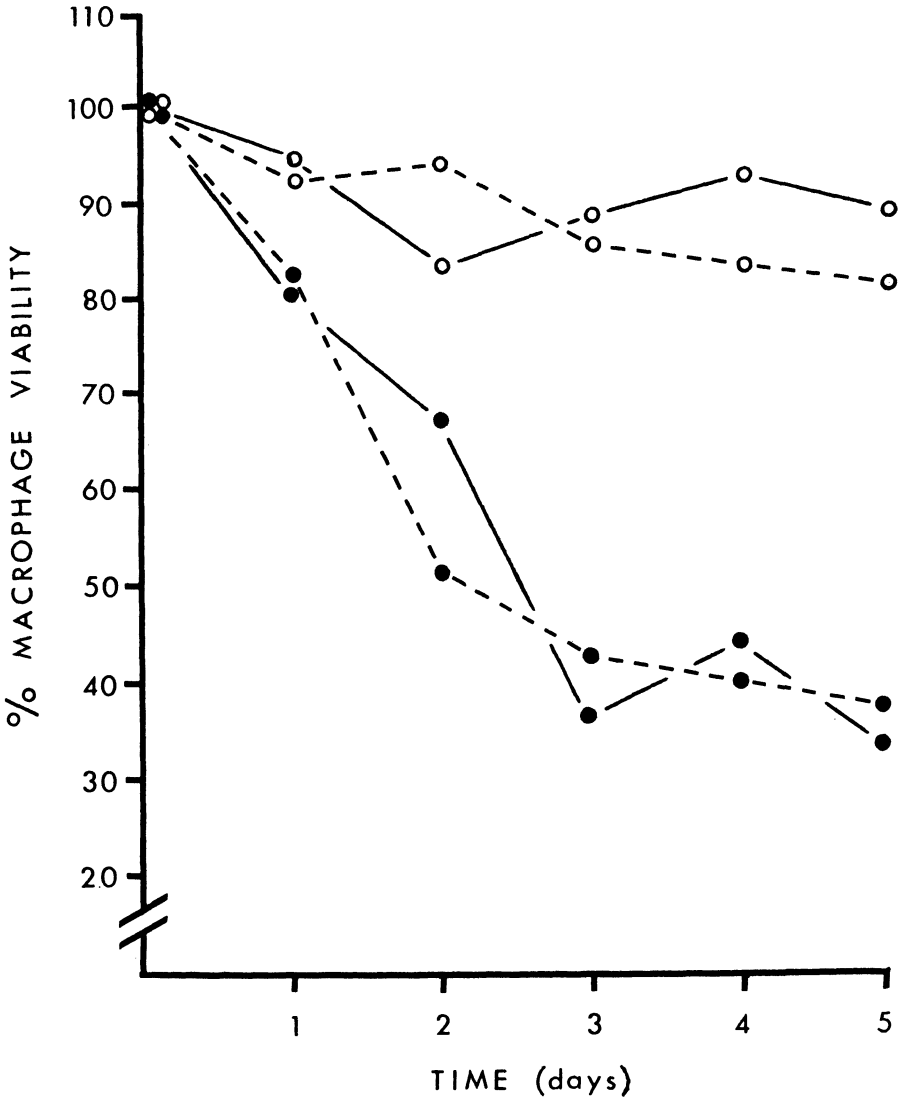


Fig. 8. Viability of normal mouse peritoneal macrophages, cultured in medium containing normal vs immune rabbit serum, and inoculated with F. tularensis strain Schu S4. Inoculated ● ; noninoculated o ; normal serum in medium - - - - - ; immune serum in medium ———— .

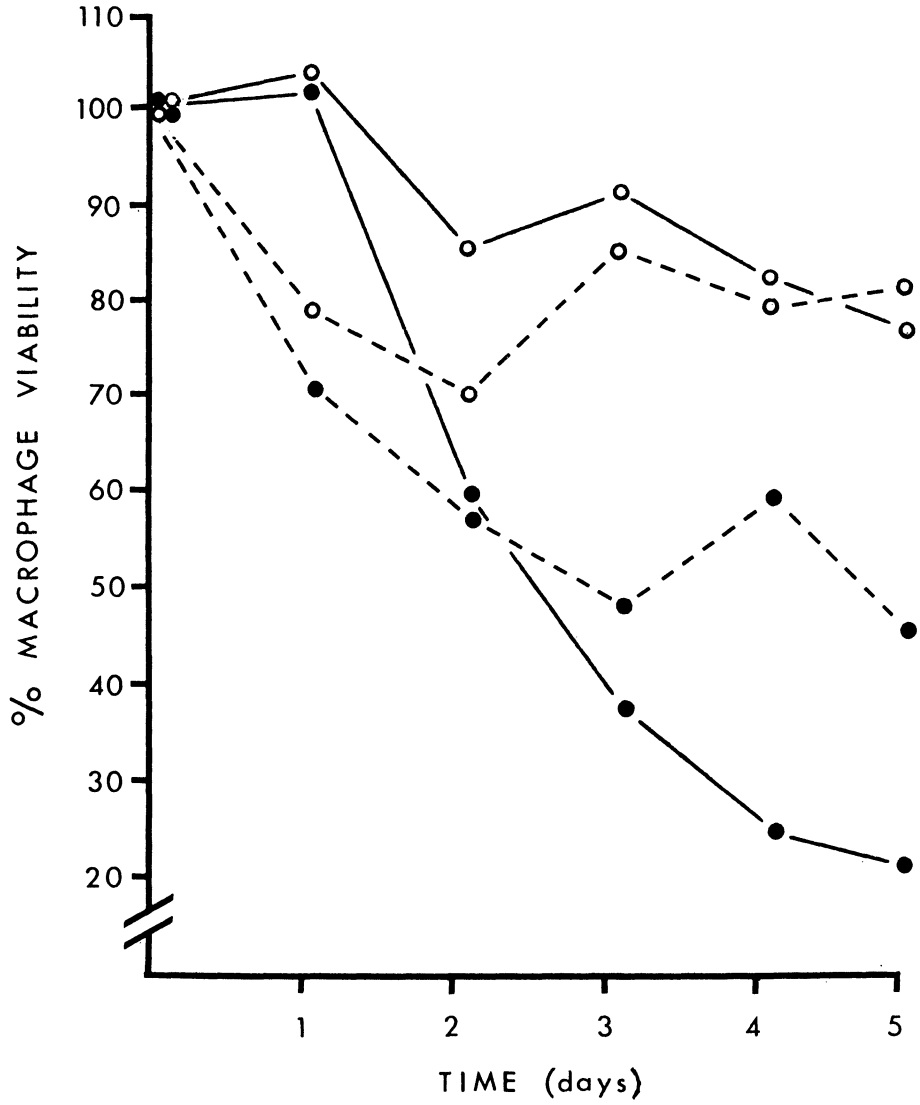


Fig. 9. Viability of macrophages from BCG immunized mice, cultured in medium containing normal vs immune rabbit serum, and inoculated with F. tularensis strain Schu S4. Inoculated ● ; noninoculated o ; normal serum in medium - - - - - ; immune serum in medium ———— .

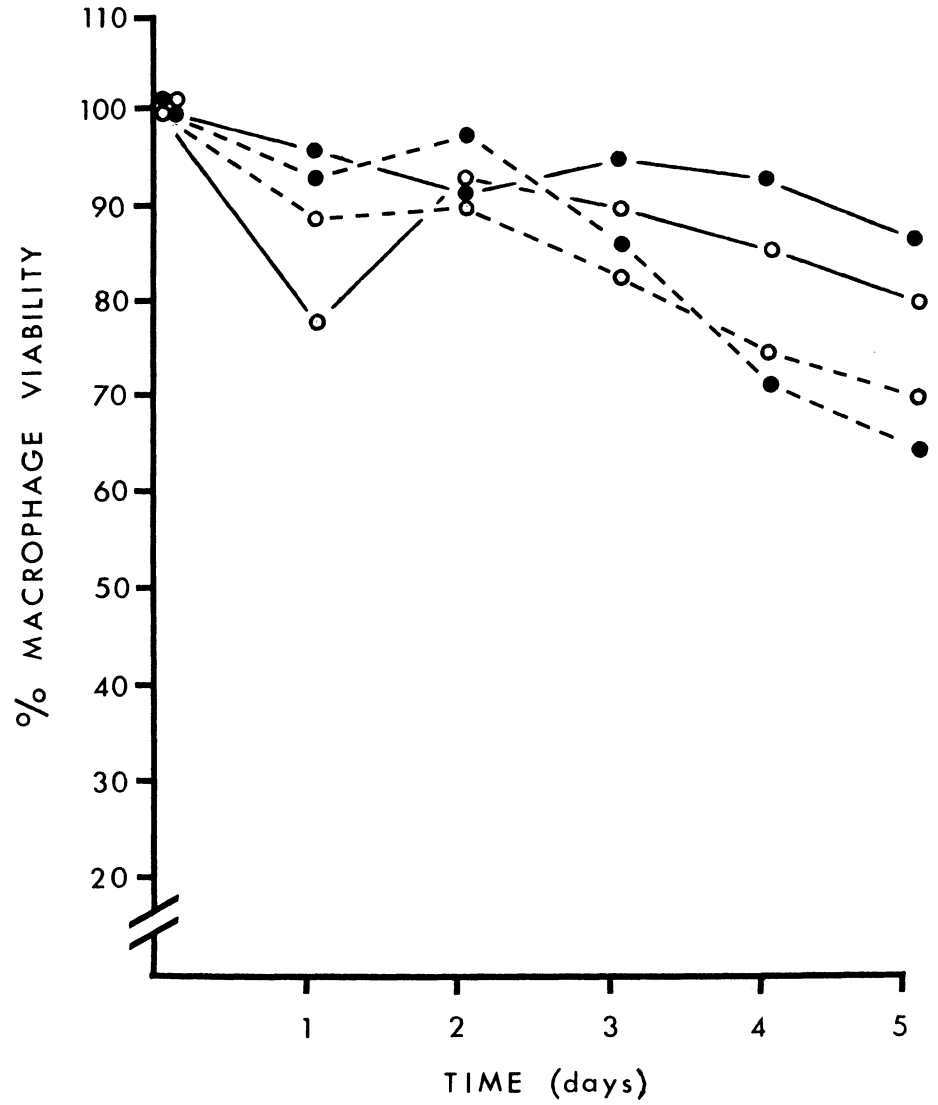


Fig. 10. Viability of macrophages harvested from mice specifically immunized with viable F. tularensis, cultured in medium containing normal vs immune rabbit serum, and inoculated with F. tularensis strain Schu S4. Inoculated ● ; noninoculated o ; normal serum in medium - - - - - ; immune serum in medium ——— .

Experiment 2. - Determination of macrophage cytopathology via ^{51}Cr-release after inoculation of cultures with F. tularensis strain Schu S4.

The previous cell count method for determining the effect of F. tularensis strain Schu S4 on cultured macrophages was considered to be excessively crude. A quicker, simpler, more sensitive and more reproducible method for assaying cytopathology was applied, viz ^{51}Cr-release.

Cultures of ^{51}Cr-labelled macrophages were inoculated with F. tularensis strain Schu S4 at a ratio of approximately 10 bacteria per macrophage. The amount of label released from the cells was monitored at 12-hr intervals fro 5 days by removing the culture medium, inserting the culture vessel into a gamma counter and measuring the amount of radioactivity emitted.

Tables IX-XII and Fig. 11-13 show the effect of the various macrophage source/sera combinations on macrophage cytopathology. The loss of label from cells in noninoculated cultures is due to the natural permeability of the macrophage cell membrane and to radioisotopic decay.

Divergence in the rate of ^{51}Cr-release between control and inoculated cultures indicates that F. tularensis strain Schu S4 had a cytopathic effect on macrophages harvested from normal and BCG immunized mice. This divergence was not noted among macrophages harvested from mice which were specifically immunized with viable F. tularensis.

The presence of immune serum in the culture medium did not mitigate macrophage cytopathology. In fact, specific antibodies increased the susceptibility of normal and activated macrophages to bacterial destruction. This increase of susceptibility was greater among activated macrophages.

Table IX. Effect of F. tularensis strain Schu S4 on the release of ^{51}Cr from normal mouse peritoneal macrophages cultured in medium containing normal vs immune rabbit serum.

Hours post-inoculation	Normal serum		Immune serum	
	Control	Inoculated	Control	Inoculated
0	104,747[a]	97,109	105,864	103,841
12	83,081	80,256	82,918	83,443
24	75,994	72,591	73,597	75,994
36	69,087	65,204	66,270	59,747
48	63,892	53,937	60,683	43,767
60	59,191	40,349	57,126	29,239
72	55,964	29,381	52,903	20,548
84	54,154	24,277	49,867	16,976
96	51,317	22,137	48,372	15,016
108	47,163	19,642	46,229	11,799
120	42,977	16,335	42,326	8,823

[a]CPM remaining in adhered macrophages.

Table X. Effect of F. tularensis strain Schu S4 on the release of ^{51}Cr from macrophages harvested from BCG immunized mice, and cultured in medium containing normal vs immune rabbit serum.

Hours post-inoculation	Normal serum		Immune serum	
	Control	Inoculated	Control	Inoculated
0	165,297[a]	162,011	231,215	233,248
12	128,317	131,764	189,477	188,216
24	109,759	116,722	162,754	154,365
36	100,629	105,432	145,464	112,699
48	91,432	91,503	131,813	61,496
60	85,694	66,732	119,684	35,835
72	79,453	42,825	105,326	24,330
84	78,124	34,075	102,305	21,436
96	76,863	30,861	100,876	18,426
108	73,149	27,102	98,347	14,872
120	69,588	22,914	94,166	11,003

[a]CPM remaining in adhered macrophages.

Table XI. Effect of F. tularensis strain Schu S4 on the release of ^{51}Cr from macrophages harvested from mice specifically immunized with F. tularensis, and cultured in medium containing normal vs immune rabbit serum.

Hours post-inoculation	Normal serum		Immune serum	
	Control	Inoculated	Control	Inoculated
0	123,134[a]	136,005	214,023	206,383
12	90,797	100,974	168,789	164,615
24	83,910	93,141	138,108	141,229
36	73,605	83,750	118,948	120,970
48	66,970	75,786	108,859	109,002
60	62,426	68,831	97,860	97,513
72	59,116	64,247	93,360	93,426
84	56,327	60,823	89,706	89,552
96	52,712	56,952	85,474	84,216
108	48,223	51,263	81,892	80,070
120	45,271	47,030	77,857	75,409

[a]CPM remaining in adhered macrophages.

Table XII. The effect of various macrophage source/serum combinations on the rate of release of ^{51}Cr from macrophages in cultures inoculated with F. tularensis strain Schu S4.

Hours post-inoculation	Normal macrophages				BCG immune macrophages				F. t. immune macrophages			
	Normal serum		Immune serum		Normal serum		Immune serum		Normal serum		Immune serum	
	Contr.	Inoc.	Contr.	Inoc.	Contr.	Inoc.	Contr.	Inoc.	Contr.	Inoc.	Contr.	Inoc.
0	100.0[a]	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
12	79.3	82.6	78.3	80.4	77.6	81.3	81.9	80.7	73.7	74.2	78.9	79.8
24	72.6	74.8	69.5	73.2	66.4	72.0	70.4	66.2	68.1	68.5	64.5	68.4
36	66.0	67.1	62.6	57.5	60.9	65.1	62.9	48.3	59.8	61.6	55.6	58.6
48	61.0	55.5	57.3	42.1	55.3	56.5	57.0	26.4	54.4	55.7	50.9	52.8
60	56.5	41.6	54.0	28.2	51.8	41.2	51.8	15.4	50.7	50.6	45.7	47.2
72	53.4	30.3	50.0	19.8	48.1	26.4	45.6	10.4	48.0	47.2	43.6	45.3
84	51.7	25.0	47.1	16.3	47.3	21.0	44.2	9.2	45.7	44.7	41.9	43.4
96	49.0	22.8	45.7	14.5	46.5	19.0	43.6	7.9	42.8	41.9	39.9	40.8
108	45.0	20.2	43.7	11.4	44.3	16.7	42.5	6.4	39.2	37.7	38.3	38.8
120	41.0	16.8	40.0	8.5	42.1	14.1	40.7	4.7	36.8	34.6	36.4	26.5

[a]Percent ^{51}Cr remaining in adhered macrophages.

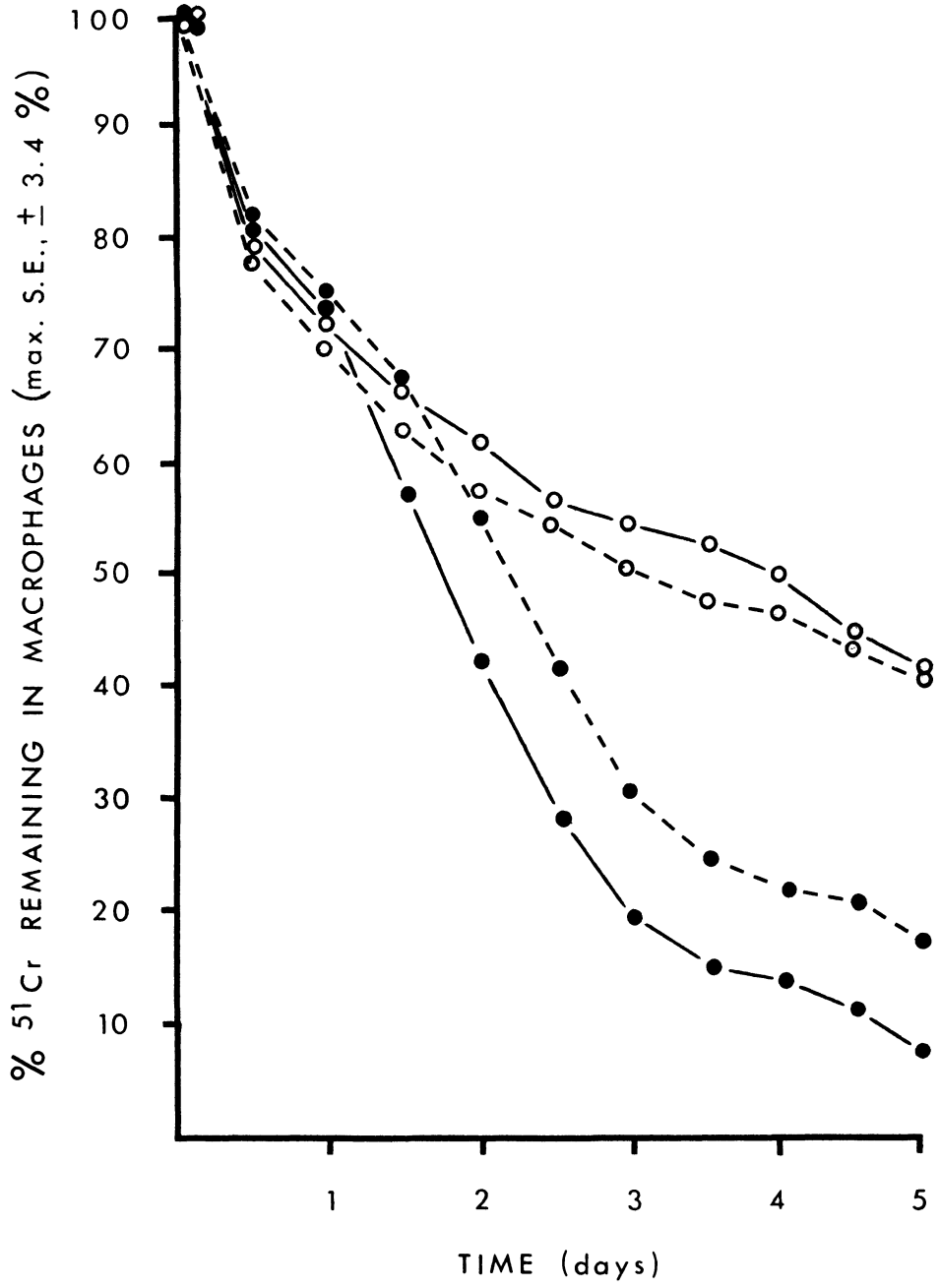


Fig. 11. Cytopathic effect of F. tularensis strain Schu S4 on macrophages harvested from normal mice, and cultured in medium containing normal vs immune rabbit serum. Inoculated ● ; noninoculated o ; normal serum in medium - - - - - ; immune serum in medium ————— .

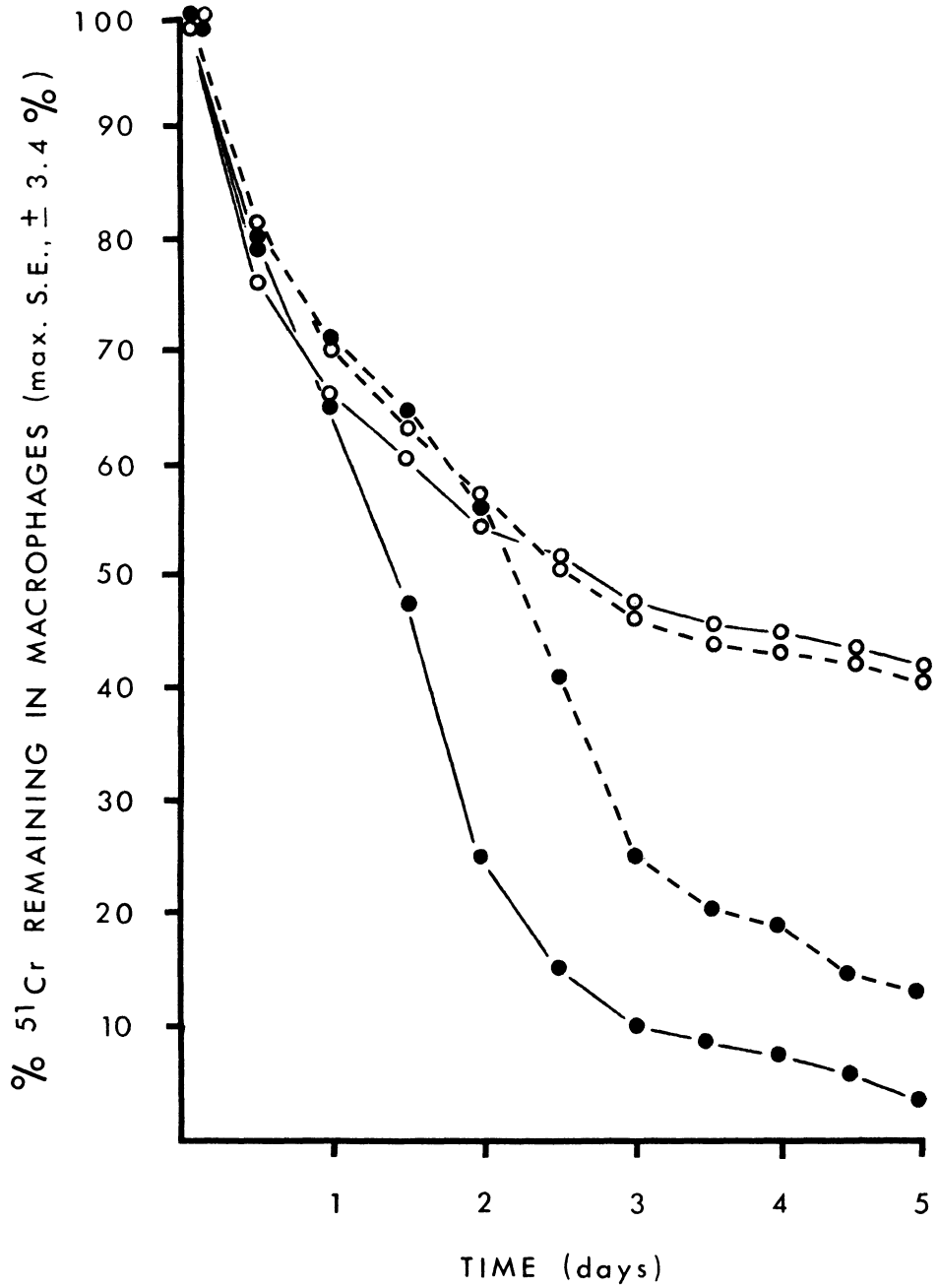


Fig. 12. Cytopathic effect of F. tularensis strain Schu S4 on macrophages harvested from mice immunized with viable BCG, and cultured in medium containing normal vs immune rabbit serum. Inoculated ● ; noninoculated o ; normal serum in medium - - - - - ; immune serum in medium ———— .

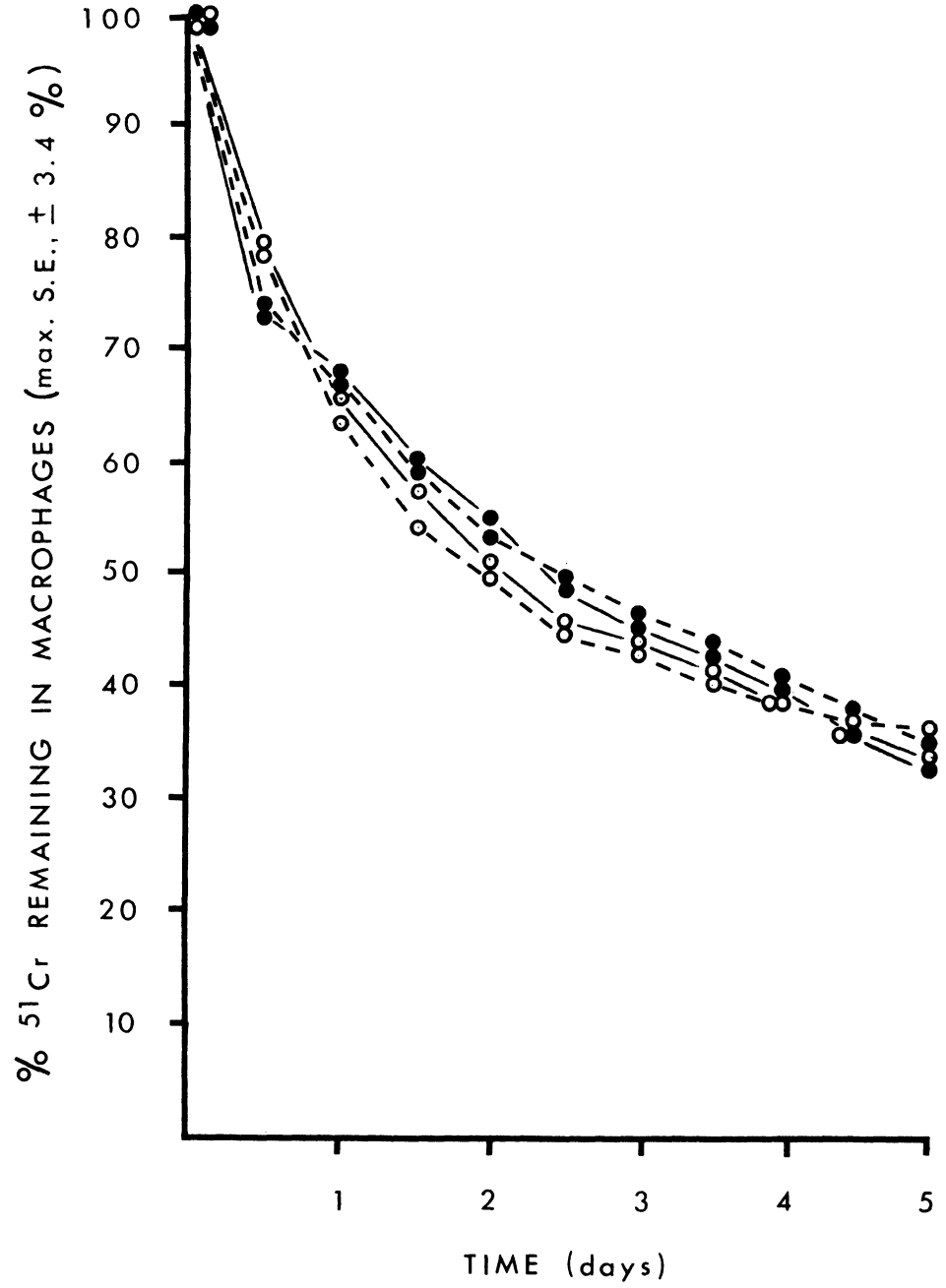


Fig. 13. Cytopathic effect of F. tularensis strain Schu S4 on macrophages harvested from mice specifically immunized with viable F. tularensis, and cultured in medium containing normal vs immune rabbit serum. Inoculated ● ; noninoculated o ; normal serum in medium - - - - - ; immune serum in medium ———— .

Experiment 3. - Growth of F. tularensis strain Schu S4 in macrophage culture.

This experiment was conducted to reconnoiter the growth kinetics of the bacterium in this particular in vitro system. Normal macrophages, cultured in medium containing normal rabbit serum, were inoculated with F. tularensis strain Schu S4 at a ratio of approximately 10 bacteria per macrophage. Also, aliquots of culture medium alone were inoculated. Bacterial growth curves were constructed by standard plate count technique.

Typical results presented in Table XIII and Fig. 14 show that the organism proliferated in the cell culture, however, the organism did not grow in the culture medium alone. Furthermore, the presence of specific antibodies in the culture medium alone, had no bactericidal effect of the organism. Other in vitro studies of F. tularensis have obtained similar results (89).

Experiment 4. - Growth of F. tularensis strain Schu S4 in cell-free macrophage culture filtrate.

Corroborative studies, which have also demonstrated an association between the presence of macrophages and bacterial growth in cell culture, have presumed that this proliferation is a consequence of intracellular multiplication (89,125). In order to determine if the bacteria are actually multiplying within the macrophage or whether it might be multiplying outside of the macrophage as a result of some possible secretory product or metabolite, serving as a nutrient, growth curves of the organism were conducted in cell-free macrophage culture filtrates.

Table XIII. Growth of F. tularensis strain Schu S4 in macrophage culture and in culture media.

Days post-inoculation	Medium containing normal serum	Medium containing immune serum	Normal macrophages in medium containing normal serum	Cell-free culture filtrate (normal serum)
0	4.1×10^4[a]	4.5×10^4	4.4×10^4	4.3×10^4
1	4.3×10^4	4.3×10^4	4.5×10^4	2.8×10^5
2	3.8×10^4	4.7×10^4	3.9×10^6	9.7×10^7
3	4.2×10^4	4.5×10^4	1.2×10^8	7.9×10^8

[a]Number of bacteria per ml.

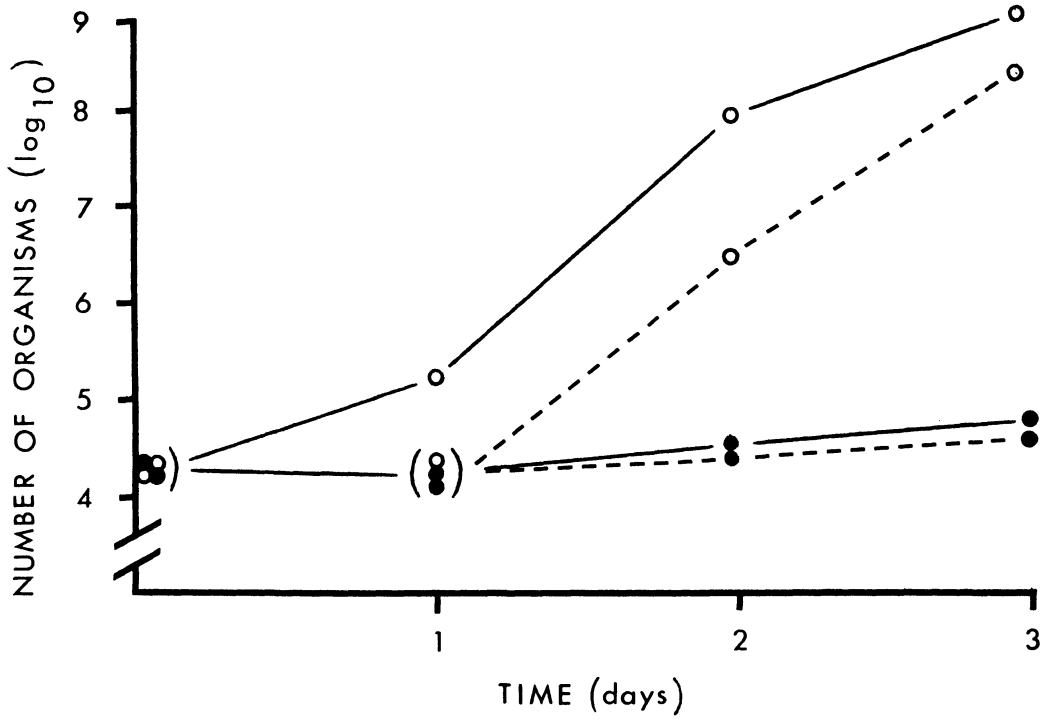


Fig. 14. Growth curves of F. tularensis strain Schu S4 in culture media and in macrophage culture. Medium & normal serum ●——●; medium & immune serum ●- - -●; normal macrophages & normal serum o---o; cell-free culture filtrate o——o.

Macrophages were harvested from normal mice and cultured in medium containing normal rabbit serum. After 3 days of incubation, the culture medium was withdrawn and filtered through a 0.22μm syringe-mounted Millipore® filter. Aliquots of the filtrate were inoculated with F. tularensis strain Schu S4 and bacterial growth curves were constructed. The results presented in Table XIII and Fig. 14 show that the bacteria proliferated in the absence of intact macrophages.

Experiment 5. - Effect of various macrophage source/serum combinations on the growth of F. tularensis strain Schu S4 in cell culture.

Macrophage cultures were set up as diagrammed in Fig. 1 and inoculated with F. tularensis strain Schu S4. Bacterial growth curves were then constructed.

Table XIV and Fig. 15 show the data from a typical experiment. Although the type of serum in the culture medium had no effect on the growth of the organism when the organism was inoculated in medium alone (Expt. 3), there was a significant difference when macrophages were present in the medium. Presence of specific antibodies in inoculated cell cultures suppressed bacterial growth. The only combination capable of completely inhibiting bacterial growth was macrophages harvested from F. tularensis immune mice, and cultured in medium containing immune serum.

Table XIV. Effect of various macrophage source/serum combinations on the growth of F. tularensis strain Schu S4 in cell culture.

Days post-inoculation	Normal macrophages		BCG immune macrophages		F. tularensis immune macrophages	
	Normal serum	Immune serum	Normal serum	Immune serum	Normal serum	Immune serum
0	4.3×10^4[a]	4.0×10^4	4.2×10^4	4.5×10^4	3.9×10^4	4.2×10^4
1	4.2×10^4	4.1×10^4	4.6×10^4	4.0×10^4	4.4×10^4	3.9×10^4
2	4.8×10^6	1.8×10^5	7.6×10^6	9.7×10^4	2.6×10^6	3.9×10^4
3	1.5×10^8	3.7×10^6	3.3×10^8	5.1×10^5	3.8×10^7	3.8×10^4

[a]Number of bacteria per ml.

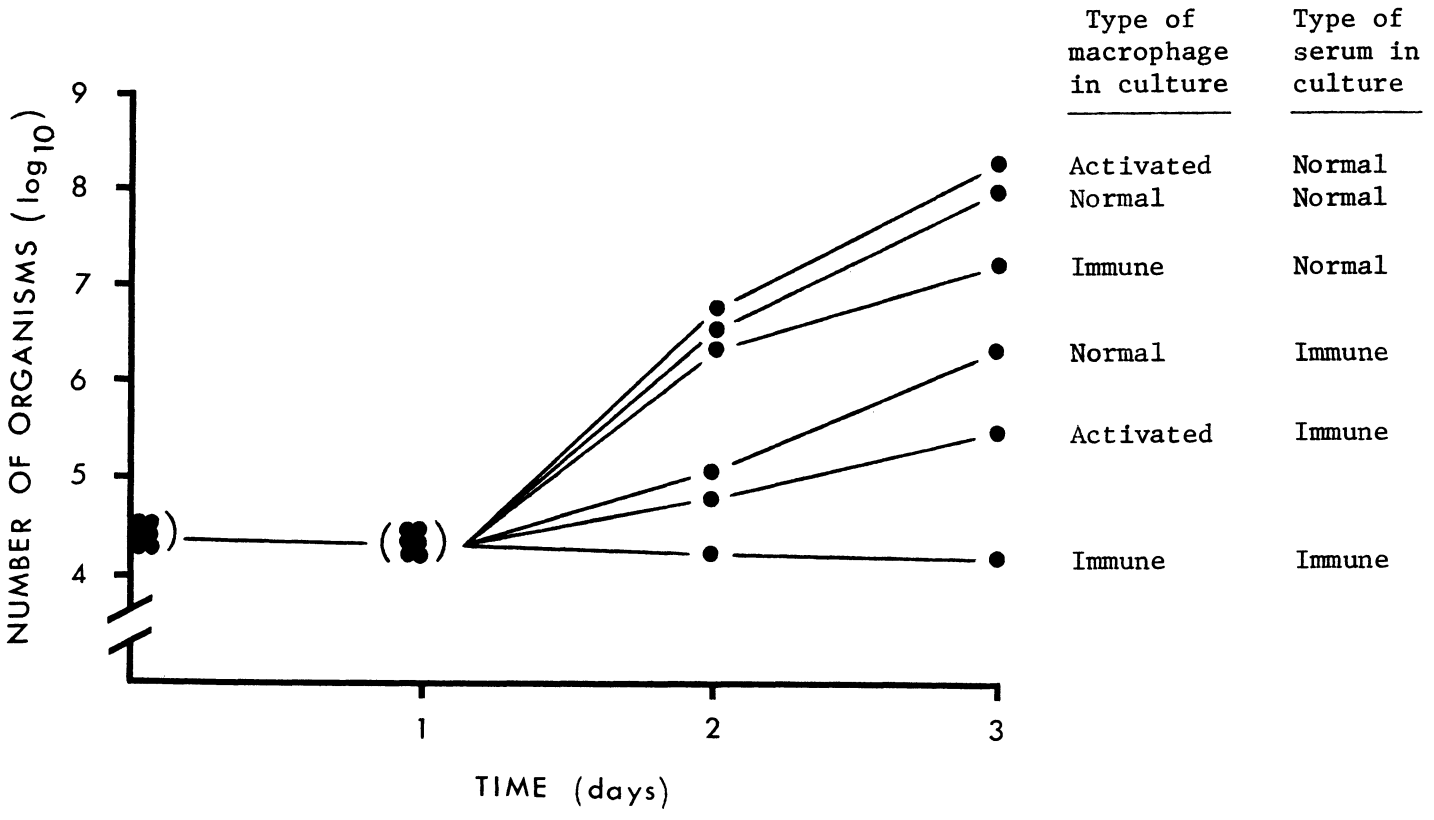


Fig. 15. Effect of macrophage source/serum combinations on the growth of F. tularensis strain Schu S4 in cell culture.

In vivo uptake of F. tularensis strain Schu S4 and L. monocytogenes by mouse peritoneal cells

Evidence has been accumulating which indicates that this organism may not be phagocytosed by peritoneal macrophages. Further investigation was conducted by determining the *in vivo* clearance of bacteria from the peritoneal cavity of mice. Suspensions of *F. tularensis* strain Schu S4 or *L. monocytogenes* were injected into the peritoneal cavities of mice. One hr after injection, the peritoneal cavities were lavaged and the exudates were separated into a cellular fraction and a supernatant fraction by centrifugation. In each case, the number of cell-associated or supernatant-associated bacteria was assayed. The data have been presented in Table XV as the percent total recoverable bacteria. It was found that *L. monocytogenes* became associated with the cellular fraction of the peritoneal exudate, whereas, *F. tularensis* remained with the fluid fraction.

To determine if antitularensis antibodies may be functioning as opsonins, groups of mice were injected with *F. tularensis* strain Schu S4 which had been pretreated with immune rabbit serum. The bacteria were incubated in the immune serum at 37 °C for 1 hr before ip. injection. This incubation was conducted at a prozone concentration of antibody to prevent bacterial agglutination. Likewise, bacteria were incubated in normal rabbit serum to serve as controls. Results are presented in Table XV. Pretreatment of the bacteria with specific antibodies caused a significant fraction of the inoculum to become cell-associated. Little interaction was noted between peritoneal cells and bacteria pretreated with normal rabbit serum.

Table XV. *In vivo* uptake of *F. tularensis* strain Schu S4 and *L. monocytogenes* by mouse peritoneal cells[a].

Challenge organism	% of total number of organisms recovered	
	Cell associated	Supernatant associated
F. t. Schu S4	7.0 ± 4.3	93.0 ± 4.3
L. monocytogenes	96.0 ± 1.2	4.0 ± 1.2
F. t. Schu S4 pretreated with normal rabbit serum	3.6 ± 1.0	96.4 ± 1.0
F. t. Schu S4 pretreated with immune rabbit serum	21.8 ± 9.8	78.2 ± 9.8

[a]Mice were challenged ip. One hour later their peritoneal cavities were lavaged, and the exudate separated into a supernatant fraction and a cellular fraction by centrifugation.

Exploratory studies were conducted to determine if strains of F. tularensis of differing virulences might differ in their association with the peritoneal cells or fluid. It was found that there was no significant difference in bacterial uptake attributable to the virulence of the organism (data not presented).

An exploratory experiment was also conducted to investigate the possibility that F. tularensis strain Schu S4 contained an antiphagocytic substance. This was done by injecting a mixed inoculum of F. tularensis strain Schu S4 and L. monocytogenes and determining if the presence of F. tularensis would modify the uptake of the Listeria. Bacterial plate counts of the respective exudate fractions were performed on a selective medium which did not support the growth of F. tularensis. It was found that the uptake of L. monocytogenes by mouse peritoneal cells was not altered by the presence of F. tularensis strain Schu S4 (data not presented).

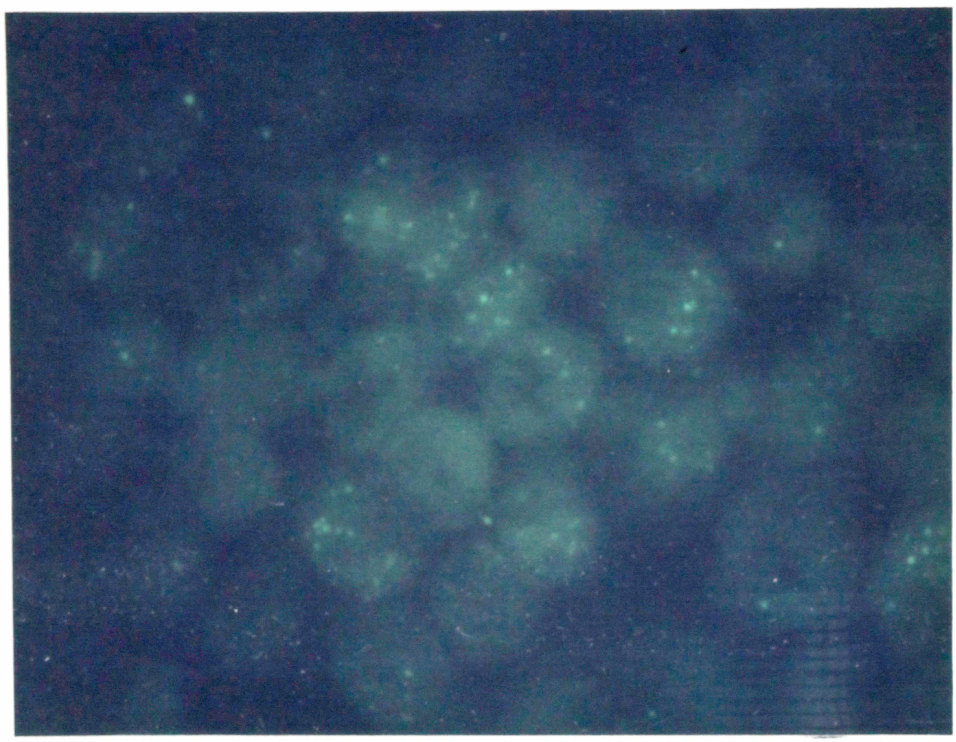
Fluorescent antibody staining of macrophages in cultures inoculated with F. tularensis strain Schu S4

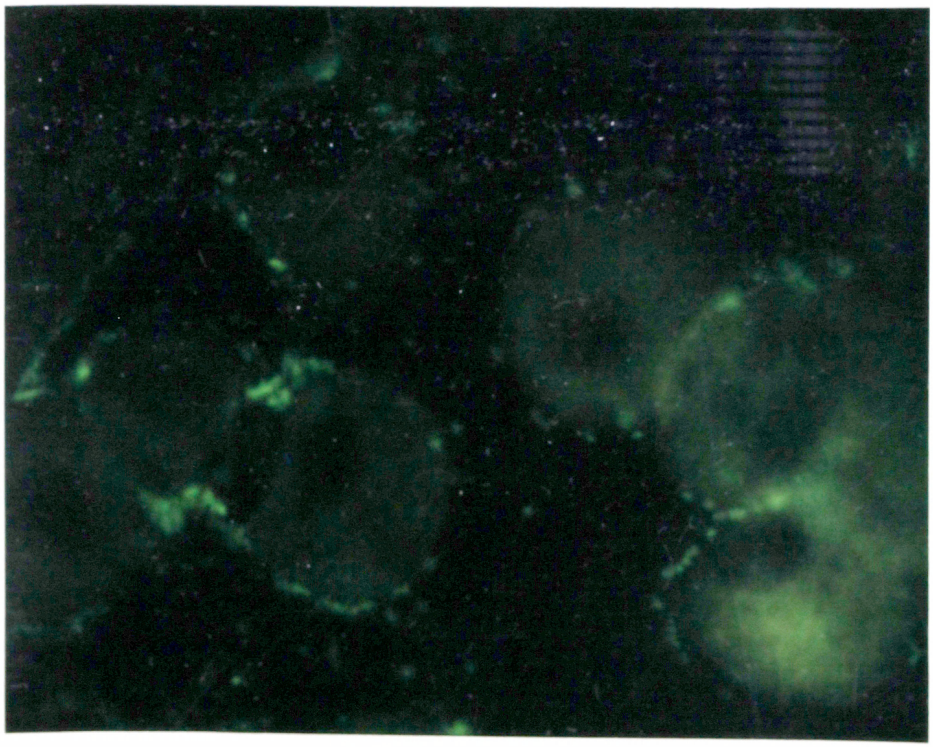
The existence of any intracellularly-located bacteria was probed by the use of fluorescein isothiocyanate-conjugated anti-tularensis immunoglobulin. As positive controls, macrophage cultures were inoculated with suspensions of formalin-killed Coxiella burnetii and incubated for 2 days. The cultures were then washed 3 times with sterile saline, fixed in acetone at -70 °C for at least 12 hr and stained with a homologous fluorescent conjugate. The presence of C. burnetii antigen within the cells was readily detected (Fig. 15).

Macrophage cultures were inoculated with F. tularensis strain Schu S4 and incubated until cytopathology was apparent. The cells were processed as described above and stained. Fluorescent microscopic examination did not reveal the presence of the organisms within the macrophages (Fig. 16). There was considerable fluorescence, however, along the external periphery of the cells. Staining of cells in noninoculated cultures showed that this peripheral fluorescence was not due to nonspecific adherence of the conjugate to the macrophage cell membrane.

Fig. 16. Mouse peritoneal adherent cells maintained in RPMI medium containing 20% rabbit serum, 2 days after exposure to formalin-killed C. burnetii, stained by fluorescent antibody technique, showing intracellular localization of antigen. 750X.

Fig. 17. Mouse peritoneal adherent cells maintained in RPMI medium containing 20% rabbit serum, 2 days after exposure to viable F. tularensis strain Schu S4, stained by fluorescent antibody technique, showing the absence of intracellular localization of the organisms. 1250X.





CHAPTER IV

DISCUSSION

The results obtained in this investigation indicate that Francisella tularensis is in extracellular association with host mononuclear phagocytes during the disease process in the mouse. The first line of evidence supporting an extraphagocytic nature of this organism is its indifference to an increased bactericidal capacity of an activated RES. In this study, viable BCG was used to activate macrophages and to induce nonspecific resistance. It has been shown that this induction resulted in significant increases in protection against known facultative intracellular parasites, but not against F. tularensis strain Schu S4. If F. tularensis is such an intracellular parasite, it is inconceivable that the increased bactericidal capacity of the activated macrophages, which resulted in over a 10^4-fold increase in protection against challenge of mice with L. monocytogenes, should not be able to even mitigate tularemic infection.

Further evidence which indicates an extraphagocytic nature of this organism is its lack of association with mouse peritoneal cells in vivo. L. monocytogenes is known to be a facultative intracellular organism. As expected, L. monocytogenes became associated with the cellular portion of the exudate when injected into the peritoneal cavities of mice. F. tularensis strain Schu S4, however, remained with the fluid portion of the exudate. Taplits and Myrvik (124) obtained similar results using F. tularensis strain LVS. These

findings tend to suggest that an antiphagocytic substance might be present on the bacterial cell wall. Proctor et al. (102) assessed phagocytosis of F. tularensis by rhesus monkey peripheral polymorphonuclear neutrophils (PMN) by the use of autoradiography, electron microscopy and biochemical techniques. They reported that F. tularensis was not ingested by PMN in the absence of immune serum and considered the possibility that this evasiveness was due to an antiphagocytic substance on the surface of the bacterium. They were unable to establish the existence of such a substance. In this laboratory, attempts to demonstrate an antiphagocytic substance were also unsuccessful. The presence of F. tularensis strain Schu S4 did not modify the uptake of L. monocytogenes by mouse peritoneal cells in vivo.

Additional evidence of lack of intraphagocytic occurrence of F. tularensis strain Schu S4 was obtained by direct fluorescent microscopic examination of inoculated cultures. Fluorescent antibody staining of moribund peritoneal macrophages in inoculated cultures did not reveal the presence of intracellular antigen. Demonstration of intracellular C. burnetii antigen with a homologous conjugate indicates that this technique was capable of detecting antigens so located. Also, staining of bacterial smear controls showed that the conjugate did indeed have a specificity for F. tularensis. A fluorescent haze was observed along the external periphery of numerous macrophages. It was found that this was not due to non-specific adherence of the conjugate to the macrophage. Functional testing of macrophages by latex particle ingestion revealed that

these cells were avidly phagocytic. Adherence of bacteria to macrophages without subsequent ingestion would be intriguing. Such an occurrence, however, would be inimical with the results obtained in the *in vivo* uptake experiment. A similar phenomenon was reported by Merriott *et al.* (85) in *F. tularensis*-infected mouse L cells. They noted that this occurrence was influenced by the concentration of serum in the culture medium. The effect of serum concentration was not investigated in this study. Although the observance of peripheral fluorescence requires more investigation, emphasis is placed on the fact that the organisms were not detected inside of the macrophages. Other studies, however, have detected *F. tularensis* within various types of host cells by means of fluorescent antibody technique (55,85,134).

From the results of this study alone, an assertion that *F. tularensis* strain Schu S4 is a strict extracellular parasite would be an unwarranted generalization. The evidence of extracellular association by *F. tularensis* presented in this investigation may apply only to mouse peritoneal macrophages. It is quite possible that the phagocytic avidity of mouse peritoneal macrophages for *F. tularensis* strain Schu S4 may differ from other animal species, or even from other cell types within the mouse. Extrapolation to other animal models and/or cell systems may not be supportable. All the evidence presented in this study, however, indicates that *F. tularensis* strain Schu S4 is in extracellular association with mouse peritoneal macrophages.

Scientific literature contains numerous references which are responsible for the belief that F. tularensis is a facultative intracellular parasite. Nutter and Myrvik (89) reportedly have grown the organism in cultured alveolar macrophages. Thorpe and Marcus (125) infected cultures of alveolar and peritoneal macrophages from rabbits and guinea pigs with various strain of F. tularensis and observed the bacteria within the cultured cells. Proctor et al. (102) incubated rhesus monkey peritoneal macrophages with ^{3}H-labelled LVS organisms and observed intracellular localization of the label. White et al. (134) have provided revealing fluorescent photomicrographs of monkey alveolar macrophages containing F. tularensis strain Schu S4 in the cytoplasm of the phagocytes.

It must be stated that, although there is good evidence of an intraphagocytic nature of this organism in animal species other than the mouse and in cell types other than peritoneal cells, such evidence is scanty as far as the mouse peritoneal macrophage system is concerned. The possibility of a unique phagocytic avidity among mouse peritoneal macrophages has been suggested by Thorpe and Marcus (126). Findings presented in this study are not inconsistent with the literature.

Studies which have been cited in support of an intracellular nature of this organism have often been presumptuous in assuming that bacterial proliferation and cytopathology are a consequence of intracellular multiplication. Nutter and Myrvik (89) inoculated suspensions of rabbit peritoneal macrophages with F. tularensis

strain Schu S4 and noted that the bacteria proliferated. They stated that this increase was "most probably" the result of intracellular multiplication. Actual phagocytosis of the bacteria has often only been inferred, rather than proved. Thorpe and Marcus (125) determined the concentration of bacteria within phagocytes in vitro, and also ingestion and digestion rates of the bacteria, by light microscopy. The small size (0.2-0.3 x 0.7 μm) and the extreme pleomorphism of this organism, coupled with the granularity of the macrophage cytoplasm, appears to this writer, and also to others (83,102), to preclude identification of the organism within phagocytes by conventional light microscopy.

Correlation has been dimonstrated in this study between bacterial growth in cell culture medium and the presence of macrophages. The in vitro system of Nutter and Myrvik (89) produced similar findings. An association between bacterial growth in cell culture medium and the presence of macrophages does not necessarily imply that the bacterial growth is occurring within the macrophage. The location of proliferation was investigated in this study by monitoring the growth of the organism in macrophage culture filtrates. The organism was found to grow in the cell-free culture filtrate as luxuriantly as in the cell culture. It is astonishing that none of the studies reviewed by this writer have considered the possibility of this occurrence. Apparently, macrophages in this system released some excretory product or metabolite which provided a growth requirement for this fastidious bacterium. The existence of such extracellular macrophage products is well established (6, 97,117, 131,133). Limited characterization of this factor was conducted.

It was heat stable (56 °C for 30 min). Bacterial growth curves in culture media containing varying dilutions of the filtrate were constructed. It was found that as the concentration of the filtrate decreased, the rate of growth of the bacteria decreased and also that the number of organisms eventually attainable decreased. This suggests that the nature of this factor is not vitamin-like, but rather, that it may be a consumable product. Further characterization is beyond the scope of this investigation. The important point is that the correlation which can be shown between bacterial proliferation and the presence of macrophages in this system, may be explainable in terms of extracellular growth.

Numerous studies have recently been reported which also suggest an extraphagocytic nature of this organism. Proctor et al. (102) found that F. tularensis resisted phagocytosis by human, monkey and rat PMN in vitro. They postulated that this phagocytic evasiveness deleted one of the possible host defense mechanisms and permitted early dissemination of the organism to specific target tissues. Taplits and Myrvik (124) observed that Listeria were rapidly eliminated from the peritoneal cavity of mice challenged ip., whereas F. tularensis strain LVS maintained a steady state population. They suggested that F. tularensis may be able to grow extracellularily and eventually overcome the host defense machanisms. Powanda et al. (99) studied metabolic sequelae induced in rats by infection with F. tularensis strain Schu S4. They concluded that "F. tularensis in nonimmune rats eludes the RES."

The subject of humoral involvment in resistance to tularemia remains an area of controversy. Specific antibodies have generally been believed to be only of secondary importance in immunity to murine tularemia. Passive transfer of serum from resistant mice has repeatedly failed to protect normal recipients against challenge with fully virulent strains of F. tularensis (70,80). Claflin (20) found that specific immune serum from mice immunized with LVS was incapable of protecting mice from even a low challenge dose of F. tularensis strain Schu S4. Furthermore, increasing the titer and the quantity of the antiserum did not produce resistance, and led him to conclude that "specific antibodies, per se, are not of primary importance in immunity to tularemia." Also, opsonization of the bacteria prior to challenge was not found to increase resistance. Peckinpaugh (95) observed that although comparable levels of antibodies were present in groups of mice immunized with nonviable and viable vaccines, the resistance of the 2 groups of mice to infection with fully virulent strains of F. tularensis was not comparable. Thorpe and Marcus (125,126) reported that the type of serum (normal vs immune) in cell culture media did not significantly affect the ingestion of F. tularensis by rabbit or guinea pig peritoneal macrophages in vitro. Similar findings were reported by Nutter and Myrvik (89). Serum antibodies are not always an indication of the immune state.

Studies cited in the preceeding paragraph discount the importance of humoral immunity in host resistance to infection with F. tularensis. The results presented here, conflict with those findings. This study presents evidence that antitularensis immuno-

globulins may have an important role in the expression of immunity in mice. This may seem in contradiction with the finding that the establishment of a specific humoral response in mice by active or passive immunization did not alter the course of infection by F. tularensis strain Schu S4, a finding in agreement with numerous other studies (20,21,95). However, further investigation revealed some interesting insights. In conjunction with an activated RES, specific antibodies resulted in measurable protection against challenge with F. tularensis strain Schu S4. Luoma (70,71) conducted similar experiments with F. tularensis strain 425 F_4G and also found that a combination of humoral and cellular factors resulted in protection. This increase in protection was only minor, and of no practical significance to the host, but, nevertheless, it does indicate that antibodies may participate in an immune response to this organism. The finding that the presence of specific antibodies in cell culture media resulted in differences in cytopathology of mouse peritoneal macrophages in inoculated cultures also indicates humoral involvement. Destruction of normal and BCG immune macrophages by F. tularensis strain Schu S4 was greater when the cultures contained media with immune serum. This finding was unexpected. It is interesting that specific antibodies can be shown to increase the susceptibility of host cells to a pathogen. This will be discussed later. Antibody involvement was also indicated by an inhibitory effect of immune serum on bacterial growth in cell culture. When bacteria were inoculated into medium in the absence of macrophages, it was found that the immune serum in the culture medium did not

inhibit bacterial growth any more than normal serum. When macrophages were present in the culture medium, however, immune serum resulted in a general suppression of bacterial growth in comparison with normal serum. Although there are indications of a protective action of antibodies during tularemic infection in mice, the exact nature of this interaction is uncertain.

Other studies have also challenged the contention that antibodies are unimportant in resistance to tularemia. Luoma (70,71) found that cyclophosphamide, an immunosuppressive agent effective in eliminating the antibody producing cell, abolished immunity to F. tularensis strain 425 F_4G. She concluded that immunity to F. tularensis results from a conbination of specific and nonspecific immunity. Proctor et al. (102) found that specific antibodies were required for the phagocytosis of F. tularensis by human, monkey and rat PMN. Taplits and Myrvik (124) monitored the growth of F. tularensis strain LVS in the organs of infected mice and found that the decline of the LVS population in these mice coincided with the appearance of specific agglutinins. Eigelsbach et al. (35) showed that adaptive transfer of spleen cells from Schu S4 vaccinees was often protective only when antitularensis serum was also administered.

In this study, evidence has been obtained which suggests that the antibodies may be activg as opsonins. It was found that the presence of immune serum in the medium of inoculated cultures increased the destruction of nonimmune macrophages by the bacteria. A similar phenomenon was also observed by Thorpe and Marcus (126).

One possible explanation is that the antibodies opsonized the bacteria. Opsonization resulted in the phagocytosis of the bacteria by the macrophages. Having been phagocytosed, the bacteria were placed in a position where the phagocytes were more vulnerable to the cytocidal activity of the bacteria. It was also found that immune serum enabled the macrophage cultures to more effectively inhibit the growth of this organism in cell cultures. Bacterial growth was suppressed in cultures containing immune serum. The growth curves of the organism in medium containing normal vs immune serum without macrophages, demonstrated that this suppression was not due to complement-mediated bacteriolysis. Considering the findings that; 1.) the organisms were not detectable within the cells of inoculated macrophage cultures containing normal serum, 2.) the organisms were capable of extracellular proliferation in such cultures, and that 3.) specific antibodies by themselves were not deleterious to the viability of the organism, the suppression of bacterial growth in cell cultures containing immune serum could be explained by reasoning that the specific antibodies functioned as opsonins and resulted in the engulfment of the bacteria into the unfavorable environment within the phagocyte. Pretreatment of bacteria with homologous antibodies increased bacterial association with peritoneal cells in vivo. This confirms findings by Taplits and Myrvik (124). Although this increase was not as dramatic as hoped, it is statistically significant.

Other evidence exists which also supports an opsonic nature of antibodies in the immune response to tularemic infection. Claflin (20)

found that the clearance of virulent tularemia bacilli from the blood of mice was enhanced when specific antibodies were passively into the mice. Proctor et al. (102) demonstrated that immune serum enabled monkey PMN to engulf F. tularensis in vitro. Taplits and Myrvik (124) reported that mice had difficulty in removing F. tularensis strain LVS from the peritoneal cavity before the appearance of specific antibodies.

Resistance to tularemia has been shown to be dependent on a state of altered reactivity of the host cells. Results obtained in this investigation confirm that dependency. The exact mechanism by which host cells become specifically sensitized and exert antibacterial properties is still undefined. Only macrophages harvested from mice specifically immunized with viable tularemia organisms were found to be resistant to the cytopathic effect of F. tularensis strain Schu S4. Also, only macrophages from this source were capable of completely inhibiting bacterial growth. Macrophages from other sources apparently lacked some specific, altered, intrinsic quality.

Immunization with a nonviable antigen did not result in protection against challenge by fully virulent strains of F. tularensis. Van der Meer et al. (130) suggested that the inability of nonviable vaccines to protect against some infections may be due to rapid and extensive digestion of the antigen. They found that complete digestion of previously killed Listeria did not result in protection, but that this nonviable vaccine became protective when macrophage activity was impaired by the addition of polyanions. Incomplete processing of nonviable antigens could result in continued antigenic

stimulation and may be a prerequisite for protection.

The experiment which was conducted to investigate the effect of various macrophage source/serum combinations on the growth of F. tularensis strain Schu S4 in cell culture produced some revealing results which may serve to clarify and unite the results obtained elsewhere in this study. It was found that macrophages harvested from resistant mice were able to suppress bacterial growth only when specific antibodies were present in the system. It was only these macrophages which posessed the forementioned ability to inactivate ingested organisms. Although these phagocytes had the ability to destroy the organisms, this ability was ineffectual, unless antibodies were available to opsonize the organisms. In the absence of antibodies, the bacteria may not be ingested, and proliferate extracellularily and escape intraphagocytic destruction. In the presence of antibody, the bacteria are presumable opsonized and thereby placed in a position where inactivation can occur. Immunity of mice to F. tularensis strain Schu S4 thus appears to require specific sensitization of reticuloendothelial cells. This enables the host to inactivate the organism. Immunity appears to also require specific antibodies. These are necessary to expose the organisms to the bactericidal machinery in the intracellular environment of the sensitized macrophage. With this in mind: 1.) the inability of viable BCG to induce nonspecific immunity effective against challenge with this organism can be explained by reasoning that specific antibodies were absent; 2.) the inability of active immunization with EEA or passive administration of immune serum to

result in protection against infection can be explained by reasoning that the macrophages lacked the functional capacity to inactivate the ingested bacteria; 3.) the protection which resulted from the combination of RES activation and humoral immunity can be explained by reasoning that the specific antibodies opsonized the bacteria and exposed them to the increased bactericidal properties in the intracellular environment of the BCG activated macrophages; 4.) the finding that the susceptibility of normal and BCG immune macrophages to bacterial cytopathology was greater in cultures containing immune serum than in cultures containing normal serum can be explained by reasoning that the specific antibodies in the immune serum positioned the bacteria in a location where the macrophages are more vulnerable to the cytocidal activity of the bacteria and finally, 5.) inhibition of bacterial growth in macrophage culture can be expected to occur only in cultures containing immune serum and cells harvested from mice specifically immunized with viable organisms.

The cytopathology which was noted in inoculated cultures should be accountable. Intracellular bacterial proliferation leading to rupture of the macrophage is improbable in light of the lack of any evidence of intracellular existence of the organisms in this cell type (in the absence of opsonins). Some bacterial factor directed against the macrophage is indicated. F. tularensis is not known to produce any toxins, although there are reports that viable F. tularensis organisms have toxic properties (11,39,64,88,89). Bacterial lipopolysaccharides are known to be cytotoxic to macrophages

however, no classical endotoxin has been demonstrated in F. tularensis. The possibility of a bacterial product toxic to macrophages in this system was briefly investigated by adding cell-free bacterial culture filtrates to macrophage cultures and observing for any resulting cytotoxicity. ^{51}Cr-release methodology showed that this bacterial culture filtrate did have a slight cytotoxic effect on the cultured macrophages. Due to a time limitation, further investigation was not conducted. Clearly, more research into this matter would be in order.

Although the phagocytic leucocyte has been implicated as the receptor cell for many facultative intracellular bacteria, evidence presented in this study indicates that this may not be the case regarding F. tularensis strain Schu S4 in the mouse. Tularemic lesions, including hemorrhagic necrosis of the spleen, focal necrosis of the liver and widespread degenerative tissue changes, clearly indicate cellular destruction in the disease process. F. tularensis is known to infect various cell types on non-lymphoid origin. Merriott et al. (85) found that F. tularensis strain Schu S4 was highly infectious for mouse L cells, bovine kidney cells and human amnion cells in vitro, and that rapid bacterial multiplication occurred in the cytoplasm of these various cells. Powanda et al. (99) maintain that F. tularensis strain Schu S4 in nonimmune rats infects the parenchymal cells of the liver and spleen. Hall et al. (55) and White et al. (134) challenged rhesus monkeys with aerosols of F. tularensis and demonstrated by immunofluorescence that the

organisms were present in the epithelial cells of the respiratory bronchioles and adjacent alveoli. Downs et al. (29) have shown that the organisms multiply abundantly in the tissues of embryonated chicken and duck eggs. Shepard (114) has shown that F. tularensis is engulfed by HeLa cells. It may well be that such non-lymphoid cells comprise the primary target tissues in the disease process during tularemia in the mouse.

The anomalies between the in vitro findings in this study and other similar studies may be due, in part, to variable inherent to such in vitro systems. Many studies have employed macrophages which were obtained from inflammatory exudates stimulated by various types of chemotactic agents or irritants. The phagocytosis of organisms by peritoneal macrophages has been shown to be influenced by these procedures (24,38). McElree and Downs (83) demonstrated that macrophages exhibited a strikingly increased ability to ingest F. tularensis when irritants were used to elicit the exudates. In this study, however, non-inflammatory cells, harvested from untreated peritoneal cavities, were used, so as to simulate more closely, the in situ physiological state of the cells. The source of animal serum best suited for the culture and parasitization of phagocytes also remains an open question (85). Guinea pig and calf sera have been reported to enhance significantly the phagocytosis of F. tularensis by peritoneal macrophages in vitro, whereas, rabbit serum failed to enhance ingestion of the organisms (83). Shepard (112) showed that HeLa cells are more phagocytic and more susceptible to infection with

tubercle bacilli when cultured in mediua containing horse serum than in media containing human serum. These variables might account for the different results obtained in this study.

Virulence factors of F. tularensis are obscure. The results of this study shed little light on this issue. It appears, though, that virulence can not be equated with the degree of association of the organisms with host phagocytes. In vivo uptake experiments have shown that avirulent and virulent strains are equally dissociated from mouse peritoneal macrophages. Other studies which have used cells capable of normally engulfing this organism have demonstrated that strains of varying virulences are equally phagocytosed (85). It is personally felt that virulence may be a function of the organisms metabolism and nutritional requirements. Strains of reduced virulence have been observed to grow more slowly on plate culture. Plate counts of such strains are lower and irregular and colonial characteristics vary considerably. Less virulent strains have also been noticed to be more sensitive to their environment. It is set forth that the virulence of F. tularensis is not associated with phagocytic evasiveness, but rather, that virulence of the organism parallels its ability to persist and multiply in the host, either intracellularly or extracellularly.

Evidence has been presented in this investigation which indicates that F. tularensis strain Schu S4 may not ordinarily be ingested by reticuloendothelial cells in the nonimmune mouse. Without phagocytosis, the organisms might be able to proliferate extra-

phagocytically, disseminate to sites of specific target tissues and eventually overcome other host defense mechanisms.

Resistance in mice to tularemic infection apparently reauires the cooperation of both humoral and cellular immune mechanisms. Host resistance may depend on the functional capacity of the host cells to inactivate the organism. Resistance may also require the opsonic effect of specific antibodies so that inactivation can be accomplished.

Host immunity, and survival of parasites, depend on a complex and delicate balance of host-parasite interplay. The mechanisms of immunity which arise in the host to rid it of intracellular parasites are not fully understood. The results of this study, although inconclusive, may serve to clarify some of the enigmas which have persisted over the years regarding host-parasite interaction during tularemia.

CHAPTER V

SUMMARY

This study has presented evidence which indicates that F. tularensis is in extracellular association with host mononuclear phagocytes during the disease process in the mouse. The course of infection of mice with F. tularensis strain Schu S4 was not mitigated by the increased bactericidal capacity of a BCG activated RES. When injected into the peritoneal cavity of mice, F. tularensis strain Schu S4 became associated with the fluid portion of the peritoneal exudate, whereas, L. monocytogenes, when injected ip. became associated with the cellular portion of the exudate. Fluorescent antibody staining of macrophage cultures inoculated with F. tularensis strain Schu S4 did not reveal the presence of intracellular organisms. This evasiveness does not appear to be due to an antiphagocytic substance on the bacterial cell surface. It is postulated that in the nonimmune mouse, F. tularensis strain Schu S4 may not ordinarily be ingested by phagocytes and may be able to overcome host defenses while in extracellular association with the host.

Contrary to the generally-held belief that specific antibodies ar only of secondary importance in immunity to tularemia, evidence is presented in this study which indicates that antibodies may have a vital role in resistance in mice. Humoral immunity in conjunction with a nonspecifically activated RES resulted in a degree of protection against challenge with F. tularensis strain Schu S4.

^{51}Cr-release assays showed that immune serum influenced the cytopathic effect of F. tularensis strain Schu S4 on cultured macrophages. Pretreatment of bacteria with immune serum increased their association with peritoneal cells in vivo. Cultured macrophages were able to suppress bacterial growth only when specific antibodies were present in the culture medium. Specific antibodies may function in host resistance by promoting phagocytosis and controlling dissemination of the infecting organisms.

Resistance in mice to tularemic infection appears to require the synergistic cooperation of both humoral and cell-mediated mechanisms of immunity. Cellular sensitization is required for inactivation of the organisms. Specific antibodies are also necessary to aid ingestion, so that inactivation can occur.

LITERATURE CITED

1. Allen, W. P.]962. Immunity against tularemia: Passive protection of mice by transfer of immune tissues. J. Exp. Med. 115:411-420.

2. Anacker, R. L., E. Ribi, D. F. Tammina, L. Fadness, and R. E. Mann. 1969. Relationship of footpad sensitivity to purified protein derivatives and resistance to airborne infection with Mycobacterium tuberculosis of mice vaccinated with mycobacterial cell walls. J. Bact. 100:5]-57.

3. Anderson, F. D., R. N. Ushijima, and C. L. Larson. 1974. Recurrent herpes genitalis: Treatment with Mycobacterium bovis (BCG). Obstet. Gynecol. 43:797-805.

4. Armstrong, A. S., and C. P. Sword. 1964. Cellular resistance to listeriosis. J. Inf. Dis. 114:258-264.

5. Barclay, W. R., R. Anacker, W. Brehmer, and E. Ribi. 1967. Effects of oil-treated mycobacterial cell walls on the organs of mice. J. Bact. 94:1736-1745.

6. Bast, Jr., R. C., R. P. Cleveland, B. H. Littman, B. Zbar, and H. J. Rapp. 1974. Acquired cellular immunity: Extracellular killing of Listeria monocytogenes by a product of immunologically activated macrophages. Cell. Immunol.]0:248-259.

7. Bell, J. F. 1945. The infection of ticks (Dermacentor variablis) with Pasteurella tularensis. J. Inf. Dis. 76:83-95.

8. Bell, J. F., C. L. Larson, W. C. Wicht, and S. S. Ritter. 1952. Studies on the immunization of white mice against infections with Bacterium tularense. J. Immunol. 69:515-524.

9. Bell, J. F., C. R. Owen, and C. L. Larson. 1955. Virulence of Bacterium tularense: A study of Bacterium tularense in mice, guinea pigs and rabbits. J. Inf. Dis. 97:162-166.

10. Bell, J. F., and S. J. Stewart. 1975. Chronic shedding tularemia nephritis in rodents: Possible relation to occurrence of Francisella tularensis in lotic waters. J. Wildlife Dis. 97: 162-166.

11. Bell, J. F. (in press)

12. Blanden, R. V., G. B. Mackaness, and F. M. Collins. 1966. Mechanisms of acquired resistance in mouse typhoid. J. Exp. Med. 124:585-600.

13. Blanden, R. V. 1968. Modification of macrophage function. J. Reticuloendothel. Soc. 5:179-202.

14. Blanden, R. V., M. J. Lefford, and G. B. Mackaness. 1969. The host response to Calmette-Guerin bacillus infection in mice. J. Exp. Med. 129:1079-1091.

15. Boehme, D., and R. J. Dubos. 1958. Effects of bacterial constituents on resistance of mice to heterologous infection and on activity of the reticulo-endothelial system. J. Exp. Med. 107:523-536.

16. Buchanan, R. E., and N. E. Gibbons, (eds.). 1974. Bergey's Manual of Determinative Bacteriology, 8th Ed. Baltimore: Williams & Wilkins.

17. Buchelle, L., and C. M. Downs. 1949. Studies on pathogenesis and immunity in tularemia. II. Immune response of white rats to Bacterium tularense. J. Immunol. 63:135-145.

18. Burroughs, A. L., R. Holdenried, D. S. Longanecker, and K. F. Meyer. 1945. A field study of latent tularemia in rodents with a list of all known naturally infected vertebrates. J. Inf. Dis. 76: 115-119.

19. Burrows, W. 1973. Textbook of Microbiology, 20th Ed. Philadelphia: W. B. Saunders Co.

20. Claflin, J. L. 1970. Immunogenicity of viable and nonviable Francisella tularensis vaccines. Ph.D. Thesis, 119 pp., University of Montana.

21. Claflin, J. L., and C. L. Larson. 1972. Infection-immunity in tularemia: Specificity of cellular immunity. Inf. Imm. 5; 311-318.

22. Collins, F. M., G. B. Mackaness, and R. V. Blanden. 1966. Infection-immunity in experimental salmonellosis. J. Exp Med. 124:601-619.

23. Collins, F. M., and G. B. Mackaness. 1968. Delayed hypersensitivity and arthus reactivity in relation to host resistance in Salmonella-infected mice. J. Immunol. 101:830-845.

24. Conrad. R. E., L. C. Yand, and H. B. Herscowitz. 1977. Mononuclear phagocytic cells in peritoneal exudates of rabbits: A comparison of inducing agents. J. Reticuloendothel. Soc. 21:103-107.

25. Cooper, G. N., and A. E. Stuart. 1966. Susceptibility of mice to pneumococcal infection after modification of the RES with simple lipids. J. Path. Bact. 83:227-243.

26. Cornforth, J. W., P. Hart, R. J. Reese, and J. R. Stock. 1951. Antituberculous effect of certain surface active polyoxyethylene ethers in mice. Nature 168:150-153.

27. Dahlstrand, S., O. Ringertz, and B. Zetterberg. 1971. Airborne tularemia in Sweden. Scand. J. Inf. Dis. 3:10-15.

28. Dannenberg, A. M. 1968. Cellular hypersensitivity and cellular immunity in pathogenesis of tuberculosis: Specificity, systemic and local nature, and associated macrophage enzymes. Bact. Rev. 32:85-102.

29. Downs, C. M., L. L. Coriell, S. S. Chapman, and A. Klauber. 1947. The cultuvation of Bacterium tularense in embryonated eggs. J. Bact. 53:89-100.

30. Downs, C. M., L. L. Coriell, H. T. Eigelsbach, K. F. Plitt, G. B. Pinchot, and B. J. Owen. 1947. Studies on tularemia. II. Immunization of white rats. J. Immunol. 56:229-243.

31. Downs, C. M., and J. M. Woodward. 1949. Studies on pathology and immunity in tularemia. III. Immunogenic properties of various strains of Bacterium tularense for white mice. J. Immunol. 63: 147-163.

32. Dubos, R. J., and R. W. Schaedler. 1957. Effects of cellular constituents on the resistance of mice to heterologous infections. J. Exp. Med. 106:703-717.

33. Eigelsbach, H. T., and C. M. Downs. 1961. Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccines and evaluation in the white mouse and guinea pig. J. Immunol. 87:415-425.

34. Eigelsbach, H. T., J. J. Tulis, M. H. McGavran, and J. D. White. 1962. Live tularemia vaccine. I. Host-parasite relationships in monkeys vaccinated intracutaneously or aerogenically. J. Bact. 84:1020-1027.

35. Eigelsbach, H. T., D. H. Hunter, and W. A. Janssen. 1974. Mechanisms of host resistance to tularemia. J. Reticuloendothel. Soc. 16, 36a (Abstr.).

36. Elberg, S. S., P. Schneider, and G. Fong. 1957. Cross-immunity between Brucella melitensis and Mycobacterium tuberculosis. Intracellular behavior of B. melitensis in monocytes from vaccinated animals. J. Exp. Med. 106:545-554.

37. Elberg, S. S. 1960. Cellular immunity. Bact. Rev. 24:67-95.

38. Elberg, S. S., P. Mascarenhas, and J. Fong. 1960. Response of peritoneal exudate cells to Brucella melitensis. Influence of nature of inflammatory irritant. Proc. Soc. Exp. Biol. Med. 104:295-297.

39. Finegold, M. J., J. D. Pulliam, M. E. Landay, and G. G. Wright. 1969. Pathological changes in rabbits injected with Pasteurella tularensis dilled by ionizing radiation. J. Inf. Dis. 119: 635-640.

40. Fong, J., P. Schneider, and S. S. Elberg. 1956. Studies on tubercle bacillus-monocyte relationship. I. Quantitative analysis of effect of serum of animals vaccinated with BCG upon bacterium-monocyte system. J. Exp. Med. 104:455-465.

41. Fong, J., P. Schneider, and S. S. Elberg. 1957. Studies on tubercle bacillus-monocyte relationship. II. Induction of monocyte effect on resistance to degeneration. J. Exp. Med. 105:25-37.

42. Foshay, L. 1932. Prophylactic vaccination against tularemia. Am. J. Clin. Path. 2:7-10.

43. Foshay, L., W. H. Hesselbrock, H. J. Wittenberg, and A. H. Rodenberg. 1942. Vaccine prophylaxis against tularemia. Am. J. Pub. Health 32:1131-1145.

44. Foshay, L. 1950. Tularemia. Ann. Rev. Micro. 4:313-330.

45. Francis, E. 1921. Tularemia. I. The occurrence in nature as a disease of man. Pub. Health Rep. 361731-1738.

46. Francis, E. 1937. Sources of infection and seasonal incidence of tularemia in man. Pub. Health Rep. 52:103-113.

47. Frenkel, J. K. 1967. Adaptive immunity to intracellular infection. J. Immunol. 98:1309-1319.

48. Frobisher, M., R. D. Hinsdill, K. T. Crabtree, and C. R. Goodheart. 1974. Fundamentals of Microbiology, 9th Ed. Philadelphia: W. B. Saunders Co.

49. Gaspar, A. J., H. B. Tresselt, and M. K. Ward. 1961. New solid medium for enhanced growth of Pasteurella tularensis. J. Bact. 82:564-569.

50. Gaugas, J. M., and R. J. W. Rees. 1968. Enhancing effect of antilymphocyte serum on mycobacterial infection in mice. Nature 219:408-409.

51. Goiman-Yahr, R., and S. Ferraresi. 1969. Delayed hypersensitivity in relation to suppression of growth of Listeria monocytogenes by guinea pig macrphages. J. Bact. 100635-640.

52. Golub, E. S. 1977. The Cellular Basis of the Immune Response: An Approach to Immunobiology. Sunderland, Mass.: Sinauer Assoc. Inc.

53. Gordon, M. 1962. Studies on the relationship of delayed hypersensitivity in immunity to tularemia. Inf. Imm. 90:209-216.

54. Guerrant, R. L., M. K. Humphries, Jr., J. E. Butler, and R. S. Jackson. 1976. Tickborne oculoglandular tularemia: Case report and review of seasonal and vectorial associations in 106 cases. Arch. Intern. Med. 136:811-813.

55. Hall, W. C., R. M. Kovatch, and R. L. Schricker. 1973. Tularemia pneumonia: Pathogenesis of the aerosol-induced disease in monkeys. J. Pathol. 110:193-201.

56. Holland, J. J., and M. J. Pickett. 1958. A cellular basis of immunity in experimental Brucella infection. J. Exp. Med. 108:343-360.

57. Howard, J. G. 1961. Resistance to infection with Salmonella paratyphi C in mice paralyzed with a relatively avirulent strain of Salmonella typhimurium. Nature 191:87-88.

58. Isa, A. M. 1971. The immunosuppressive effect of rabbit anti-mouse macrophage serum. J. Immunol. 107:595-597.

59. Jenkin, C. R., and B. Benacerraf. 1960. In vitro studies on the interaction between mouse peritoneal macrophages and strains of Salmonella and Escherichia coli. J. Exp. Med. 112:363-374.

60. Jenkin, C. R., and D. Rowley. 1963. Basis for immunity to typhoid in mice and the question of "cellular immunity". Bact. Rev. 27:391-401.

61. Jenkin, C. R., D. Rowley, and I. Auzins. 1964. The basis of immunity to mouse typhoid. I. The carrier state. Aust. J. Exp. Biol. Med. Sci. 43:65-78.

62. Kadull, P. J., H. Reames, L. L. Coriell, and L. Foshay. 1950. Studies on tularemia. V. Immunization of man. J. Immunol. 65:425-435.

63. Koster, F. T., D. D. McGregor, and G. B. Mackaness. 1971. The mediator of cellular immunity. II. Migration of immunologically committed lymphocytes into inflammatory exudates. J. Exp. Med. 133:400-409.

64. Landay, M. E., G. C. Wright, J. D. Pulliam, and M. J. Finegold. 1968. Toxicity of Pasteurella tularensis killed by ionizing radiation. J. Bact. 96:804-810.

65. Larson, C. L. 1945. Immunization of white rats against infections with Pasteurella tularensis. Pub. Health Rep. 60:725-734.

66. Larson, C. L., J. F. Bell, and C. Owen. 1954. Development of resistance in mice immunized with soluble antigen derived from B. tularense. J. Immunol. 73:221-225.

67. Lemonde, P., and M. Clode. 1962. Effect of BCG infection on leukemia and polyoma in mice and hamsters. Proc. Soc. Exp. Biol. Med. 111:739-742.

68. Levey, R. H., and P. B. Medawar. 1966. Some experiments on the action of anti-lymphoid antisera. Ann. N.Y. Acad. Sci. 129: 164-177.

69. Lodmell, D. L., and L. C. Ewalt. 1978. Induction of enhanced resistance against encephalomyocarditis virus infection of mice by nonviable Mycobacterium tuberculosis : Mechanisms of protection. Inf. Imm. 22:740-745.

70. Luoma, M. K. 1973. Humoral and cellular aspects of resistance in mice to Francisella tularensis strain 425 F_4G. Masters Thesis, 79 pp., University of Montana.

71. Luoma, M. K., and C. L. Larson. 1974. Immunity to Francisella tularensis: A combination of specific and non-specific immunity. Abstracts of the annual meeting of the ASM, p 88.

72. Lurie, M. B. 1939. Studies on the mechanism of immunity in tuberculosis. The role of extracellular factors and local immunity in the fixation and inhibition of growth of tubercle bacilli. J. Exp. Med. 69:555-578.

73. Lurie, M. B. 1942. Studies on the mechanism of immunity in tuberculosis. The fate of tubercle bacilli ingested by mononuclear phagocytes derived from normal and immunized animals. J. Exp. Med. 75:247-268.

74. Mackaness, G. B. 1962. Cellular resistance to infection. J. Exp. Med. 116:381-406.

75. Mackaness, G. B. 1964. The immunological basis of acquired cellular resistance. J. Exp. Med. 120:105-120.

76. Mackaness, G. B., R. V. Blanden, and F. M. Collins. 1966. Host-parasite relations in mouse typhoid. J. Exp. Med. 124:573-584.

77. Mackaness, G. B., and R. V. Blanden. 1967. Cellular immunity. Progr. Allergy 11:89-140.

78. Mackaness, G. B. 1968. The immunology of antituberculous immunity. Am. Rev. Resp. Dis. 97:337-344.

79. Mackaness, G. B. 1969. The influence of immunologically committed lymphoid cells on macrophage activity in vivo. J. Exp. Med. 129:973-992.

80. Mackaness, G. B., and W. C. Hill. 1969. The effect of anti-lymphocyte globulin on cell-mediated resistance to infection. J. Exp. Med. 129:993-1012.

81. McCoy, G. W., and C. W. Chapin. 1912. Studies of plague, a plague-like disease and tuberculosis among rodents in California. V. Bacterium tularense-the cause of a plague-like disease of rodents. Pub. Health Bull. No. 53:17-23.

82. McCrumb, F. 1961. Aerosol infection of man with Pasteurella tularensis. Bact. Rev. 25:262-267.

83. McElree, H., and C. M. Downs. 1961. The phagocytosis of Pasteurella tularensis by rat mononuclear cells as influenced by normal serums and various irritants. J. Inf. Dis. 109:98-106.

84. McGregor, D. D., F. T. Koster, and G. B. Mackaness. 1971. The mediator of cellular immunity. I. The lifespan and circulation dynamics of the immunologically committed lymphocyte. J. Exp. Med. 133:389-399.

85. Merriott, J., A. Shoemaker, and C. M. Downs. 1961. Growth of Pasteurella tularensis in cultured cells. J. Inf. Dis. 108: 136-150.

86. Miller, J. F., and G. F. Mitchell. 1968. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. J. Exp. Med. 128:801-821.

87. Mitchell, G. F., and J. F. Miller. 1968. Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. J. Exp. Med. 128:821-831.

88. Moody, M. D., and C. M. Downs. 1955. Studies on tularemia. I. The relation between certain pathogenic and immunogenic variants of Pasteurella tularensis. J. Bact. 70:297-304.

89. Nutter, J. E., and Q. N. Myrvik. 1966. In vitro interactions between rabbit alveolar macrophages and Pasteurella tularensis. J. Bact. 92:645-651.

90. Ormsbee, R. A., J. F. Bell, and C. L. Larson. 1955. Studies on Bacterium tularense antigens. I. The isolation, purification, and biologic activity of antigen preparations from Bacterium tularense. J. Immunol. 74:351-358.

91. Ormsbee, R. A., and C. L. Larson. 1955. Studies on Bacterium tularense antigens. II. Chemical and physical characteristics of protective antigen preparations. J. Immunol. 74:359-370.

92. Osawa, N., M. Kawakami, S. Kurashige, and S. Mitsuhashi. 1967. Experimental salmonellosis. VIII. Postinfective immunity and its significance for conferring cellular immunity. J. Bact. 93:1543-1540.

93. Owen, C. R., E. O. Buker, W. L. Jellison, D. B. Lackman, and J. F. Bell. 1964. Comparative studies of Francisella tularensis and Francisella novicida. J. Bact. 87:676-683.

94. Peacock, M., W. Burgdorfer, and R. A. Ormsbee. 1971. Rapid fluorescent-antibody conjugation procedure. Inf. Imm 3:355-357.

95. Peckinpaugh, S. D. 1975. A study of the effector cell responsible for immunity in mice to Francisella tularensis strain Schu. Masters Thesis, 67 pp., University of Montana.

96. Philip, R. N., E. A. Casper, and D. B. Lackman. 1967. The skin test in an epidemiologic study of tularemia in Montana trappers. J. Inf. Dis. 117:393-402.

97. Pick, E., and J. L. Turk. 1972. The biological activities of soluble lymphocyte products. Clin. Exp. Immunol. 10:]-23.

98. Pike, R. M., S. E. Sulkin, and M. L. Schulze. 1965. Continuing importance of laboratory-acquired infections. Am. J. Pub. Health 55:190-199.

99. Powanda, M. C., P. G. Canonico, and G. L. Cockerell. 1974. Host-parasite interactions during tularemia in the rat. Fed. Proc. 33:620.

100. Procházka, O., H. Dubanská. 1972. The antigenic structure of Francisella tularensis. I. Isolation of the ether antigen and its fractionation. Folia Micro. 17:232-238.

101. Procházka, O., H. Dubanská. 1972. The antigenic structure of Francisella tularensis II. Fractionation of the ether antigen on cellulose carriers and by gel filtration. Folia Micro. 17: 300-307.

102. Proctor, R. A., J. D. White, E. Ayala, and P. G. Canonico. 1975. Phagocytosis of Francisella tularensis by rhesus monkey peripheral leucocytes. Inf. Imm. 11:146-151.

103. Quan, S. F., A. G. McManus, and H. Von Fintel. 1956. Infectivity of tularemia applied to intact skin and ingested in drinking water. Science 123: 942-943.

104. Ralston, D. J., and S. S. Elberg. 1968. Serum-mediated immune cellular responses to Brucella melitensis . I. Role of a macrophage-stimulating factor in promoting ingestion of Brucella by streptomycin-protected cells. J. Bact. 96: 24-38.

105. Ralston, D. J., and S. S. Elberg. 1969. Serum-mediated immnue cellular responses to Brucella melitensis II. Restriction of Brucella by immune sera and macrophages. J. Reticuloendothel. Soc. 6:109-139.

106. Rich, A. R. 1930. The role of allergy in tuberculosis. Arch. Intern. Med. 43:691-714.

107. Rowley, D. 1964. Endotoxin-induced changes in susceptibility to infections. In M. Landy and W. Braun (eds.), Bacterial Endotoxins. Rutgers University Press.

108. Ruskin, J., M. McIntosh, and J. S. Remington. 1969. Studies on the mechanisms of resistance to phylogenetically diverse organisms. J. Immunol. 103:252-259.

109. Saito, K., M. Nakona, T. Akiyama, and D. Ushiba. 1962. Passive transfer of immunity to typhoid by macrophages. J. Bact. 84: 500-507.

110. Saslaw, S., H. T. Eigelsbach, H. E. Wilson, J. A. Prior, and S. Carhart. 1961. Tularemia vaccine study. I. Intracutaneous study. Arch. Intern. Med. 107:688-701.

111. Senterfelt, N. C., and J. W. Shands. 1970. Salmonellosis in mice infected with Mycobacterium bovis BCG. Inf. Imm. 1: 583-586.

112. Shepard, C. C. 1955. Phagocytosis by HeLa cells and their susceptibility to infection by human tubercle bacilli. Proc. Soc. Exp. Biol. Med. 90:392-396.

113. Shepard, S., E. Ribi, and C. L. Larson. 1955. Electron microscopically revealed structural elements of Bacterium tularense and their in vitro and in vivo role in immunologic reactions. J. Immunol. 75:7-14.

114. Shepard, C. C. 1959. Nonacid-fast bacteria and HeLa cells: Their uptake and subsequent intracellular growth. J. Bact. 77 701-714.

115. Shilo, M. 1959. Nonspecific resistance to infections. Ann. Rev. Micro. 13:255-278.

116. Snyder, T. L., R. A. Penfield, F. B. Engley, Jr., and J. C. Creasy. 1946. Cultivation of Bacterium tularense in peptone media. Proc. Soc. Exp. Biol. Med. 63:26-30.

117. Sorber, W. A. 1978. Effect of complement fixation on the release of lysosomal enzymes from rabbit alveolar macrophages. Inf. Imm. 19:799-806.

118. Stansberry, M. H., and J. M. Woodward. 1962. Cellular defenses of white rats in immunity to tularemia. Abstracts of the annual meeting of the ASM. Bact. Proc. p 94.

119. Stefanye, D., H. B. Tresselt, and L. Spero. 1960. Observations. on the behavior in vitro of Pasteurella tularensis after phagocytosis. J. Bact. 81:470-473.

120. Sulitzeanu, D., A. Bekierkunst, L. Groto, and J. Loebel. 1962. Studies on the mechanism of nonspecific resistance to Brucella induced in mice by vaccination with BCG. Immunol. 5:116-128.

121. Suter, E. 1953. Multiplication of tubercle bacilli within mononuclear phagocytes in tissue cultures derived from normal animals and animals vaccinated with BCG. J. Exp. Med. 97: 235-245.

122. Suter, E. 1956. Interaction between phagocytes and pathogenic organisms. Bact. Rev. 20:94-132.

123. Suter, E., and H. Ramseier. 1964. Cellular reactions in infection. Adv. Immunol. 4:117-173.

124. Taplits, M. S., and Q. N. Myrvik. 1977. Host resistance to the LVS strain of Francisella tularensis in BCG vaccinated mice. J. Reticuloendothel. Soc. 22:297-308.

125. Thorpe, B. D., and S. Marcus. 1964. Phagocytosis and intracellular fate of Pasteurella tularensis. I. In vitro studies with rabbit peritoneal mononuclear phagocytes. J. Immunol. 92:657-663.

126. Thorpe, B. D., and S. Marcus. 1964. Phagocytosis and intracellular fate of Pasteurella tularensis. II. In vitro studies with rabbit alveolar and guinea pig alveolar and peritoneal mononuclear phagocytes. J. Immunol. 93:558-565.

127. Thorpe, B. D., and S. Marcus. 1965. Phagocytosis and intracellular fate of Pasteurella tularensis. III. In vivo studies with passively transferred cells and sera. J. Immunol. 94: 578-585.

128. Tigertt, W. D. 1962. Soviet viable Pasteurella tularensis vaccines (a review of selected articles). Bact Rev. 26: 354-373.

129. Unanue, E. R. 1968. Porperties and some uses of anti-macrophage antibodies. Nature 21:36-38.

130. Van Der Meer, C., F. M. A. Hofius, and J. M. N. Willers. 1977. Killed Listeria monocytogenes becomes protective on addition of polyanions. Nature 269:594-595.

131. Van Furth, R., (ed.). 1975. Mononuclear Phagocytes in Immunity, Infection and Pathology. Blackwell Scientific Publications.

132. Van Metre, T. E., and P. J. Kadull. 1959. Laboratory-acquired tularemia in vaccinated individuals: a report of 62 cases. Ann. Intern. Med. 50:621-632.

133. Werb, Z., and S. Gordon. 1975. Secretion of a specific collagenase by stimulated macrophages. J. Exp. Med. 142:346-360.

134. White, J. D., J. R. Rooney, P. A. Prickett, E. B. Derrenbacher, C. W. Beard, and W. R. Griffith. 1964. Pathogenesis of experimental respiratory tularemia in monkeys. J. Inf. Dis. 114:277-283.

135. Wiener, E., A. Beck, and M. Shilo. 1965. Effect of bacterial lipopolysaccharides on mouse peritoneal leucocytes. Lab. Invest. 14:475-487.

136. Woodward, J. M., M. H. Stansberry, W. G. Presswood, and J. B. Fox. 1964. Cellular immunity of rats to tularemia. Can. J. Micro. 10:573-577.

137. Youmans, G. P., and A. S. Youmans. 1969. Allergenicity of mycobacterial ribosomal and ribonucleic acid preparations in mice and guinea pigs. J. Bact. 97:134-139.

138. Young, L. S., D. S. Bicknell, and B. G. Archer. 1969. Tularemia epidemic: Vermont, 1968. Forty-seven cases linked to contact with muskrats. New Eng. J. Med. 280:1253-1260.